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Cyanobacteria species are distributed worldwide and extensive growth can result in cyanotoxin production, hypoxic zones, human health risk, and mortality of fish, domesticated animals, invertebrates, and plants. Better analytical tools can help water quality managers and regulatory agencies assess and manage cyanobacteria and cyanotoxin abundance to reduce environmental and health risk.

This study determined the presence and relative abundance of 4 potentially toxic cyanobacteria taxa (*Microcystis aeruginosa, Cylindrospermopsis raciborskii, Lyngbya wollei,* and *Aphanizomenon/ Anabaena* spp.), total cyanobacteria, and three toxin genes (microcystin *mcyH+/A*-, cylindrospermopsin *PKS*, and anatoxin *PKS*) using qPCR, and concentrations of microcystin and cylindrospermopsins in selected samples using ELISA assays. All species were found in all lakes, but abundance of individual taxa SSU rDNA and toxin genes differed among lakes. Microcystins were found at low levels (≤0.31ppb) in 4 of the 6 lakes sampled, but were not correlated with *mcyH+/A*- abundance. The anatoxin *PKS* gene was found in all lakes sampled and was significantly correlated with *Aphanizomenon/ Anabaena* spp. SSU rDNA in two of the lakes. The cylindrospermopsin *PKS* gene and cylindrospermopsins were not found in any of the lakes sampled.

# QUANTIFICATION OF SELECT CYANOBACTERIA AND CYANOTOXINS IN PIEDMONT NORTH CAROLINA LAKES USING REAL-TIME PCR

by

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Approved by

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Dedicated to my mother, Kathleen Fondario, who was a silent voice driving me forward through tough times and to my Husband, Dave Grubbs, who always gave unwavering love, support, and inspiration.

APPROVAL PAGE

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

# CHAPTER I

# INTRODUCTION

Harmful algal blooms have been reported globally, including in the United States and Canada (Fristachi and Sinclair 2008). In 1998, the Hypoxia Research and Control Act (HABHRCA) for marine and estuarine water provided funding for marine system cyanobacteria monitoring. HABHRCA was amended in 2004 to include a scientific assessment of freshwater harmful algal blooms. This amendment outlined causes for freshwater algal blooms (FWHABs) and the associated environmental and economic impacts (Hudnell *et al*. 2008). It also provided a framework for future research on cyanobacteria harmful algal blooms in freshwater systems, developed management strategies if an algal bloom should occur, and encouraged collaboration among federal agencies.

Cyanobacteria and cyanotoxins present freshwater quality problems. The United States Environmental Protection Agency (EPA) recognized cyanobacteria and cyanotoxins as drinking water contaminants and listed them on the Contaminant Candidate List 2 in 2005. Samples taken from public water systems across the US and Canada between June 1996 and January1998 exhibited the hepatotoxic microcystins in 80% of the samples. Not all water treatment plants are designed to filter cyanotoxins and thus they may persist through water treatment. Microcystins, cylindrospermopsins, and anatoxins were all found in finished drinking water in Florida in 2001. These toxins

pose a significant public health threat (Burns 2005). Consequently, by 2008, a number of states (New York, New Hampshire, Nebraska, and Florida) had implemented management strategies to monitor cyanobacteria toxins (Fristachi and Sinclair 2008).

Factors Influencing Cyanobacteria Growth

Many factors influence cyanobacteria growth. Conditions such as light availability, nutrient influx, water temperature, rainfall and lake stratification all affect the cyanobacteria communities within lakes. Each cyanobacteria species may have a different complex set of conditions it needs to survive and thrive.

Cyanobacteria reside in the photic zone of lakes and rely on photosynthesis for energy (Graham, *et.al.* 2008). *Anabaena* spp., *Microcystis* spp., *Aphanizomenon* spp., and *Cylindrospermopsis* spp. contain intracellular gas vacuoles to maintain position within the photic zone. Some species of cyanobacteria can migrate vertically from the photic to the aphotic zone. In the photic zone temperatures are often optimal for cyanobacteria growth. Most cyanobacteria grow optimally at temperatures between 20°C and 30°C (Konopka and Brock 1978), but have been recorded in temperate lakes and reservoirs at cooler temperatures. The epilimnion of a stratified temperate lake could remain at an optimal temperature for cyanobacteria growth throughout the summer (Graham *et al*. 2008).

Phosphorus loading is thought to be the overall limiting factor for cyanobacteria growth. Freshwater systems with low N: P ratios tend to support a cyanobacteria based ecosystem (Paerl 2008). Since some species, such as *Anabaena*, *Cylindrospermopsis*, *Nodularia*, and *Aphanizomenon* spp., have the ability to fix nitrogen using heterocysts,

phosphorus becomes the primary nutrient influence in promoting cyanobacteria growth. Lakes are particularly vulnerable to phosphorus nutrient influx (Paerl 2008). Phosphorus loading is high in urban areas due to increased surface run off, wastewater treatment plant effluent, fertilizer application, and municipal and industrial pollution. In rural areas septic tank drainage/leakage, soil erosion, and animal waste runoff contribute to phosphorus loading in lakes. At least 50% of the annual phosphorus loading in agricultural and urban watersheds is due to non-point sources (Paerl 2008). In systems with ample amounts of phosphorus, temperature seems to be the driving factor in cyanobacteria growth (Ahn *et. al.* 2011).

Rainfall may also effect cyanobacteria growth. Heavy rainfall can cause increased nutrients to be washed into reservoirs promoting algal growth. However, if a bloom is already established and conditions become unfavorable with increased cloudiness rates for extended periods of time or intense rainfall with increased flushing rate, cyanobacteria abundance could be lowered (Havens 2008). Increased nutrient influx from heavy rainfall followed by reduced export due to drought provides conditions for cyanobacteria growth (Paerl and Huisman 2010).

When multiple factors are conducive to cyanobacteria growth, blooms may occur (Graham, *et.al.* 2008). Blooms can occur in eutrophic, mesotrophic, and oligotrophic reservoirs as well as saltwater systems. A bloom can be dispersed throughout the water column or reside in or on surface water and can potentially form mats. Blooms are deemed harmful if they create hypoxic zones from decomposition, negatively affect surrounding plant or animal communities, or produce toxins (Paerl 2008). Hypoxic zones

can adversely affect wildlife (Butler *et. al.* 2009, Fristachi and Sinclair 2008). Dense filamentous growth on surface water can result in death of plant communities or cause physical obstruction to fish gills causing fish kills. Blooms can effect food web structure, plant structure, and/or destroy natural habitat (Fristachi and Sinclair 2008).

Cyanotoxins: Environmental and Organism Health Risks

Cyanobacteria capable of producing toxins are referred to as toxigenic cyanobacteria. These toxins can affect the liver (hepatotoxins), the brain and neuromuscular system (neurotoxins), or the skin and mucous membranes (dermatoxins). Hepatotoxins, such microcystins and cylindrospermopsins, can cause elevated liver enzymes, hepatitis, vomiting, headache, kidney damage, malaise, weakness, or anorexia (Fristachi and Sinclair 2008). Cylindrospermopsins have also been known to cause respiratory arrest in mice. Neurotoxic anatoxins can cause involuntary muscle movement, convulsions, and death. Microcystins are of particular concern because they can disrupt protein phosphatases, interfere with cell structure and mitosis, and cause liver failure and cellular death in animals. In humans, microcystins promote liver tumors (WHO 2003). Cyanobacteria blooms linked to microcystins and anatoxins have caused mortality in bird populations and cyanotoxin contaminated submerged vegetation has been linked to avian vacuolar myelopathy (Butler 2009, Fristachi and Sinclair 2008).

Polyketide synthases (*PKS*) and peptide synthetases (*PS*) are involved in the production of microcystins, cylindrospermopsins, and anatoxins. The gene that encodes microcystin *PKS* is part of the microcystin (*mcy*) gene cassette, which consists of 10 open reading frames *mcyA- mcyJ* (Kaebernick *et. al*. 2002). In *Cylindrospermopsis raciborskii*

the genes that encodes for *PKS* and *PS* are part of the cylindrospermopsin (*cyr*) gene cluster and are involved in the activation of pathways and synthesis of molecules that are needed to make cylindrospermopsins (Pearson *et. al.* 2010). The genes that encode for the anatoxin *PKS* are part of the anatoxin biosynthesis pathway (*ATX*) (Ballot *et. al.* 2010).

Organization of the *mcy* gene cassette may vary among species (Figure 1) and the promoter for these genes may also be in different locations. For example, the *Microcystis aeruginosa mcy* gene cassette has a light dependent bidirectional central promoter region that transcribes two polycistronic operons: *mcy*ABC and *mcy*DEFHIJ (Kaebernick *et. al.* 2002, Christiansen *et. al.* 2003). In contrast, *Planktothrix* spp. have one unidirectional promoter that transcribes all *mcy* genes in a continuous transcript.

Toxigenic and non-toxigenic variants may exist within a species (Fristachi and Sinclair 2008). Even if a cyanobacteria species has the genes to produce toxins they may not be active. In *Planktothrix* spp., if the *mcyT* gene is inactivated the cyanobacteria survive but lose the ability produce microcystins (Christiansen *et. al.* 2008). There was a 94 ±2% reduction in the microcystin production in *Planktothrix* spp. with the *mcyT* gene knocked out. Christiansen *et. al.* (2008) also found that the loss of the *mcyT* gene, in *Planktothrix* spp., eventually causes inactivation, leaving remnants of the *mcy* genes, or total deletion of *mcy* genes (Figure 2).

Cyanotoxins, such as microcystins and cylindrospermopsins, can bioaccumulate in consumers. Xie *et. al.* (2005) found microcystins in higher concentrations in the livers and muscles of piscivorous fish than in phytoplanktivorous or herbivorous fish.

Cylindrospermopsins are found in highest concentrations in lower trophic level organisms, such as bivalves and gastropods and lower concentrations in amphibians and fish (Kinner 2010). However, cylindrospermopsin toxicity is actually higher in fish and aquatic vertebrates, likely due to differences in toxin metabolism among invertebrates and vertebrates.

Cyanobacteria blooms can present health risks to humans. Cases of gastrointestinal discomfort, respiratory distress, fatigue, swimmers itch, skin rashes, joint pain, and nausea have been linked to cyanobacteria (Hudnell and Dortch 2008, Fristachi and Sinclair 2008). Patients in Brazil experienced visual impairment, vomiting, intestinal bleeding, bloating, blindness, jaundice, and acute liver failure that were linked to cyanobacteria (Pouria *et. al.* 1998). Investigators found unfiltered reservoir water containing cyanotoxins in the dialysis tubing of these patients. Of the 126 patients who reported illness 60 died. Microcystins were also found in the blood and livers of the patients (Pouria *et. al.* 1998, Butler *et. al.* 2009).

Exposure limits have been established for microcystin and cylindrospermopsin. The exposure limit for microcystin in recreational waters is 20 ppb (20 $\mu g\ L^{-1}$) (WHO 2003). The tolerable daily intake for microcystin is 0.04 $\mu g\ kg^{-1}$ body weight. This was based on acute toxicity tests in mice and on a 60 kg adult consuming 2.0 L of contaminated water daily. The tolerable daily intake for cylindrospermopsins is 0.02 g $kg^{-1}$ body weight (NIEHS 2000). Based on acute toxicity tests in rats guideline values were suggested to be 0.11 g $L^{-1}$ for a 5kg infants consuming 0.75 L water, 0.16 g $L^{-1}$ for a 10 kg child consuming 1.0 L water, and 0.48 g $L^{-1}$ for 60 kg adult consuming 2.0 L water.

Temperature may influence toxin production. When toxigenic *Cylindrospermopsis raciborskii* was grown at its optimal growth temperature cylindrospermopsin production was undetectable, although at a lower temperature cylindrospermopsins were produced (Saker and Griffith 2000). *Anabaena* spp. and *Aphanizominon* spp. produce the highest amount of anatoxin-a at 20°C (Rapala *et. al.* 1993). In *Anabaena* spp. and *Microcystis* spp., microcystins were produced in highest abundance when temperatures were between 18°C and 25°C (WHO 1999).

Cyanobacteria in North Carolina and the Piedmont

Many cyanobacteria taxa are found in North Carolina lakes. These include cyanotoxin producing species, such as *Anabaena planktonica, Aphanizomenon* spp., *Microcystis aeruginosa, Cylindrospermopsis raciborskii, Lyngbya wollei* and *Nodularia* spp. (Figure 3; Glasgow and Burkholder 2003, Touchette *et. al.* 2007, Stewart 2011). Cyanobacteria comprised ≥ 90% of phytoplankton in many NC reservoirs, including Lake Brandt and Lake Mackintosh, in 2002(Glasgow and Burkholder 2003).

Touchette *et al.* (2007) found that cyanobacteria comprised 60%- 95% of total phytoplankton mass in 11 reservoirs located in the Piedmont of North Carolina. Potentially toxic species of cyanobacteria were found at densities $>10^5$ cells $ml^{-1}$ in some reservoirs. *Cylindrospermopsis raciborskii* was the most abundant potentially toxic species in these communities. He then assessed correlations between cyanobacteria abundance in North Carolina reservoirs and factors such as reservoir age, suspended solids, and total phosphorus. Old (74-80 yrs post fill) and moderately aged (19-40 yrs post fill) reservoirs had similar taxon diversity and microcystin abundance but mean

abundance was approximately 2 times higher in old versus moderately aged reservoirs. All 11 reservoirs studied were mesotrophic and there were significant Pearson correlations between cyanobacteria abundance and chlorophyll *a* ($r = 0.62$, $p = 0.008$), and total phosphorus ($r = 0.61$, $p = 0.009$).

Stewart (2011) assessed cyanobacteria diversity by grouping sequences of cyanobacteria small subunit ribosomal DNA (SSU rDNA) from six Piedmont North Carolina lakes into operational taxonomic units (OTUs) consisting of sequences that were 97.5% similar. He then designed primers to these OTUs and used qPCR to assess abundance in City Lake. There were 3 dominant OTUs in City Lake: OTU 11, 89, and 67. These three OTUs did not match any known sequences of cyanobacteria DNA in GenBank. He also found that cyanobacteria taxa peaked at different times between June and October of 2010. There were Spearman's rank correlations between cyanobacteria OTU DNA abundance and water temperature ($r_s = 0.639$, $p = 0.0032$), dissolved oxygen ($r_s = -0.454$, $p = 0.04797$) and Kjeldahl nitrogen ($r_s = 0.539$, $p = 0.0173$) City lake.

Algal blooms are an environmental concern in North Carolina. North Carolina Department of Water Quality managers reported Lake Jordan having a possible algal bloom in October of 2011, indicated by high dissolved oxygen saturation, approximately 260 fish mortalities, and high pH (NCDENR 2011). In a private pond in Cabarrus county 100 fish were reported dead: low dissolved oxygen, a previous algal bloom and decay, and the addition of copper sulfate to the water to control vegetation were listed as the reason for the fish kill. City Lake and Lake Mackintosh, water supply reservoirs in the Piedmont of North Carolina, have had odor and taste complaints related to algae blooms

(NCEMC 2006). Lake Mackintosh investigators identified *Aphanizomenon flos-aquae* as being the dominant species contributing to the odor and taste problem. Blooms in City Lake have been reported in both the summer and winter. Winter blooms are thought to be due to increased fertilizer application in the fall.

Cyanobacteria Identification

Traditionally, cyanobacteria have been identified using a fusion of bacteriological and botanical approaches (Nübel 1997). These methods relied on cultural and morphological characteristics but often gave limited indication of phylogenetic relationships (Fristachi and Sinclair 2008). Phenotypic characteristics and cell arrangement can also change when maintained in laboratory settings (Palinska *et. al.* 1996). Advancements in molecular methods, such as PCR, have led to considerable progress in identifying cyanobacteria (Nübel *et. al.* 1997, Castiglioni *et. al.* 2004). Castiglioni *et. al*. (2004) developed species specific probes that target the variable region of the cyanobacteria 16S rRNA gene (SSU rDNA). They then used universal DNA arrays to identify target cyanobacteria species. Logan *et. al.* (2009) utilized the fluorescent marker SYBR green with specific primers in order to amplify and quantify double stranded DNA using real time PCR. Quantitative real time PCR (qPCR) has been used for the identification of the dominant cyanobacteria groups in the environment as well as the dominant toxin producers (Sivonen 2007). Stewart (2011) surveyed City Lake, NC for cyanobacteria. He designed 40 primers and used quantitative PCR (qPCR) to quantify 27 operational taxonomic units (OTUs).

In this study, qPCR was used to determine presence and abundance of selected taxa in six lakes. The first aim of this study was to determine the abundance of five potentially toxic cyanobacteria taxa (*Microcystis aeruginosa, Cylindrospermopsis raciborskii, Lyngbya wollei, and Aphanizomenon/ Anabaena* spp.) SSU rDNA using qPCR. I hypothesized that all of the lakes in this study will contain all six cyanobacteria taxa and they will be present throughout the year. I also hypothesized that these lakes would have similar taxon abundance seasonal patterns. However, within a lake individual taxon temporal patterns and abundance may vary. The second aim was to quantify cyanotoxin genes: microcystin *mcyH+/A*- gene, cylindrospermopsin *PKS* gene, and the *PKS* encoding gene fragment of the putative anatoxin biosynthesis gene cluster using qPCR. I then determined if there was a correlation between these cyanotoxin gene abundances and the abundance of cyanobacteria taxa. I hypothesized that toxin genes would be present in the samples that contain potentially toxic cyanobacteria and the abundance of toxin genes will correlate with taxon abundance. Finally, I assessed microcystin and cylindrospermopsin toxin levels using enzyme linked immunosorbant assays (ELISAs) within these six lakes. Samples for ELISAs were selected based upon the presence or absence of the toxin genes. I hypothesized that toxin levels will be correlated with cyanobacteria abundance and toxin gene abundance and I predict that low levels of toxins or no toxins will be found during late fall, winter, and early spring and higher levels during the warmest parts of the year. Results will help to assess whether this approach can be useful for water quality management and regulation.

# CHAPTER II

# MATERIALS AND METHODS

## Sample Collection

Six lakes were sampled in the Piedmont of North Carolina (Figure 4). Lakes were selected to encompass a range of lake types, sizes, ages and uses (Table 1). Samples were collected between June 2011 and September 2012. Cyanobacteria abundance is highest during warmer months of the year (Graham 2008) so water samples were collected biweekly June – August. Cyanobacteria abundance is lower during cooler months of the year (Stewart 2011) so water samples were collected once a month in September, October, November, January, and March and May. Secchi depths were recorded at each sampling event. To account for possible cyanobacteria stratification in the water column, integrated water samples were collected to a depth of 1.5 times the secchi depth. All integrated water samples were taken in accordance to the U.S. Geological Survey Techniques of Water-Resources (Graham *et. al.* 2008). Samples for qPCR were transferred to amber bottles and stored on ice until returned to the University of North Carolina at Greensboro (UNCG), where 75-200 ml of the integrated water sample was filtered 25mm glass fiber filters (Whatman GFF, nominal pore size 0.7µm). These filters were then placed in 2ml of cetyltrimethylammonium bromide (CTAB) buffer (Schaefer 1997) and stored at room temperature. Water samples for ELISA assays were collected in glass test tubes and frozen (-20°C). Integrated water samples for visual

confirmation of qPCR were collected in foil wrapped polypropylene tubes and preserved with Lugol's iodine solution (Wetzel and Likens 1979).

DNA Extraction, Quantification, and qPCR

A CTAB extraction was used to extract and purify environmental sample DNA (Schaefer 1997). This chloroform-isoamyl alcohol DNA extraction method minimizes DNA shearing and has been used to at extracting and purifying DNA in environmental samples. The purified DNA that is obtained through this method has been successfully used with qPCR. DNA was quantified using a BioTek Instruments Synergy™ 2 Microplate reader.

Purified DNA was initially diluted to a concentration of 5.0 ng $\mu l^{-1}$ using TE buffer (pH 8.0) and stored at 4°C until use. Preliminary qPCR analyses were conducted in order to determine a concentration of template DNA for qPCR that minimized inhibition and resulted in sample measurements within the range of the standards. After preliminary testing, City Lake, Oak Hollow Lake, Randleman Reservoir, Lake Brandt, and Lake Mackintosh extracted DNA were then further diluted to a concentration 0.5 ng $\mu l^{-1}$ for qPCR with total cyanobacteria and most species specific primers. However, for qPCR to detect *Aphanizomenon/ Anabaena* spp. DNA concentration of template was adjusted to 0.05 ng $\mu l^{-1}$. Extracted DNA was diluted to a concentration of 0.5 ng $\mu l^{-1}$ when used for qPCR with microcystin and cylindrospermopsin toxin primers and to a concentration of 5.0 ng $\mu l^{-1}$ for all lakes for anatoxin gene primers.

qPCR was performed with an Applied Biosystems StepOne™ or StepOnePlus™ real-time PCR system. Each reaction contained: 10µl of Power Sybr® Green PCR

master mix (Applied Biosystems), 1µl of 10 µM forward primer, 1µl of 10 µM reverse primer, 8µl of sterile deionized water, and 1µl of template DNA. Each PCR plate contained triplicate negative controls and triplicate standards at three different concentrations. Lake samples were run in duplicate. All taxon specific SSU rDNA primers were run using the following run protocol: initial denaturation of 10 minutes at 94°C, 35 cycles of 15 seconds at 95°C, 30 seconds at 60°C, 1 minute at 72°C and 15 seconds at 80°C (during which fluorescence was measured). Each toxin primer set used a unique run method and temperatures as indicated in reference papers (Table 3). An 80°C step was added to the extension step (during which fluorescence was measured). Each run was followed by a melt curve analysis to confirm proper target amplification.

A general cyanobacteria primer and four cyanobacteria taxa specific primers were used to quantify total and taxon specific SSU rDNA abundance (Table 2). Toxin specific primers were used to quantify target toxin *PKS* genes (Table 3). Preliminary testing revealed that the primers used to measure *Aphanizomenon* spp. and *L. wollei* SSU rDNA may not be taxa specific (see discussion on primer specificity). Positive controls standards were run in conjunction with each primer set.

Genus specific genomic DNA was used as the standard for *M. aeruginosa* (Collected from a *Microcystis aeruginosa* bloom in Eastern North Carolina by Burkholder, NCSU), and *L. wollei* (Culture Collection Cat #151830, Carolina Biological Supply Company) qPCR runs. Clonal SSU rDNA was used as the standard for *C. raciborskii* and *Aphanizomenon/ Anabaena* spp. qPCR runs (Stewart 2011). Clonal SSU rDNA from a cyanobacteria OTU (Stewart 2011: OTU 1, 96% match to *Prochlorothrix*

spp., GenBank entry AJ007864.1) was used to in conjunction with general cyanobacteria primers to assess total cyanobacteria abundance. Three toxin specific primers pairs were used in conjunction with positive control genomic DNA standards. Genomic DNA of known toxigenic strains of *M. aeruginosa and C. raciborskii* (cultures provided by Paul Zimba Texas A&M), and *Anabaena* spp. (Culture Collection cat #151710 Carolina Biological Supply Company) were used as standard to quantify microcystin, cylindrospermopsin, and anatoxin genes. Since the DNA used for qPCR standards were from a variety of DNA sources (genus specific extracted DNA versus clonal DNA) quantitative results are valid within taxon but not across taxa or comparable to total cyanobacteria abundance.

Microcystin and Cylindrospermopsin Enzyme Linked Immunosorbant Assays

ELISAs were used to assess microcystin LR and cylindrospermopsins in collected water samples. A Quanitiplate $^{TM}$ ELISA Kit for microcystin LR (Envirologix Inc., EP 022) was used to quantify microcystin LR toxins, and a cylindrospermopsin ELISA (Abraxis, PN 522011**)** was used to detect cylindrospermopsins in water samples following manufacturer's instructions. Results were read using a BioTek Instruments Synergy$^{TM}$ 2 Microplate reader at 450nm. These ELISA assays were run on selected samples where the presence of toxin genes had been indicate by qPCR.

Visual Confirmation

Light microscopy was used to visually confirm taxa presence within preserved lake water samples. Samples with high abundance of taxon specific SSU rDNA were

selected. A Palmer counting cell was used to subjectively compare taxa seen to identification guides (Cronberg and Annadotter 2006).

Statistical Analysis

SPSS statistical software was used for all statistical analysis (SPSS Inc, Chicago, IL). Normality of the data was determined by the Shapiro-Wilk test of normality, and variance was assessed using the Levene's test of equality of error variances. Some of the data sets were not normally distributed nor had acceptable variances so the data were transformed (Ln(x + 1)) with outliers removed (Grubbs' Test), but still failed the tests for normality and variance. Simple statistics were run with the outliers removed. Outliers are shown on figures 5-9, however, as they could be an indicator of algal blooms. A nonparametric Kruskal-Wallis test was performed (outliers included) to assess distributions among lakes. The fixed factors were the lake names and year. The dependent variables were target SSU rDNA abundances from five taxon, total cyanobacteria SSU rDNA abundance, and three toxins target DNA abundances. Wilcoxon Mann-Whitney statistical analysis was run to compare distributions differences between sampling years within each lake and species.

To assess possible correlations, Spearman's rank correlation coefficients were calculated. A scatterplot was made to determine if there was a monotonic relationship between the variables being correlated. Correlations were calculated between taxon SSU rDNA abundances, between toxin gene target DNA and the dominant toxin producing species SSU rDNA, and between toxin gene target DNA and the total cyanobacteria SSU rDNA of known toxin producers studied in this experiment. A Spearman's rank

correlation coefficient was also calculated to assess the correlation between toxin target gene abundance and toxin levels.

# CHAPTER III

# RESULTS

## Cyanobacteria Abundance

All cyanobacteria SSU rDNA taxon were found in all lakes in both summer sampling seasons with higher abundances in the summer and early fall when temperatures were warmest. Abundance of individual taxon SSU rDNA during winter months was often low or absent (Figure 5-9). Mean abundance varied among lakes (Table 4). For example, *Aphanizomenon/ Anabaena* spp. SSU rDNA ranged from 0.0006- 0.27 pg $ml^{-1}$ in Belews Lake to 0.10 – 38.2 pg $ml^{-1}$ in Randleman Reservoir.

Individual taxon specific SSU rDNA abundance had similar patterns within and among lakes although the quantity varied. Belews Lake had a lower mean and peak abundance of SSU rDNA and total SSU rDNA abundances than all other lakes (Tables 4, 5, Figures 11-15) and had consistently greater secchi depths (Figure 10). Lake Mackintosh also showed significantly lower taxa specific SSU rDNA abundances for some taxa (Table 5, Figures13-14). Lake Mackintosh had significantly less *Lyngbya wollei* SSU rDNA than Oak Hollow Lake ($p = 0.008$) and Lake Brandt ($p = 0.001$) and significantly less *Microcystis aeruginosa* SSU rDNA than Oak Hollow Lake ($p = 0.012$) and Randleman Reservoir ($p < 0.0001$).

Timing of peak abundance varied among lakes and between years. For example, all taxon specific SSU rDNA abundances in Lake Brandt in 2011 peaked later in the

summer season than all other lakes. *Aphanizomenon/ Anabaena* spp. SSU rDNA abundance peaked between September and October in City Lake and Oak Hollow Lake; whereas, in Belews Lake, Lake Mackintosh, and Randleman Reservoir *Aphanizomenon/ Anabaena* spp. SSU rDNA peaked earlier between July and August of 2011(Figure 5). Some significant differences in taxa specific SSU rDNA abundances were observed in City Lake, Oak Hollow Lake, and Lake Brandt (Table 6) between 2011 and 2012. For example, total cyanobacteria SSU rDNA was significantly higher in 2011 than 2012 in Oak Hollow Lake.

Water temperature was positively correlated with taxon specific SSU rDNA abundance in the three lakes where water temperature data was available (Table 7). Water temperature was correlated with *L. wollei* SSU rDNA abundances in both City Lake ($r_s$= 0.631, p = 0.01, n = 18) and Oak Hollow Lake ($r_s$= 0.699, p = 0.01, n = 17). In Lake Mackintosh water temperature was strongly correlated with *Aphanizomenon/ Anabaena* spp. SSU rDNA ($r_s$= 0.691, p = 0.01, n = 18) and *C. raciborskii* DNA ($r_s$= 0.657, p = 0.01, n = 17).

Overall, all taxon specific SSU rDNA abundances were correlated with each other among all lakes, but not with total cyanobacteria SSU rDNA (Table 8). However, when these correlations are calculated within individual lakes some correlations between taxa and total cyanobacteria DNA were weakened and some were strengthened, but the changes vary among lakes (Table 9). When correlations within each lake were evaluated, the SSU rDNA abundance at least one taxon was correlated with total cyanobacteria SSU rDNA abundance.

Visual Confirmation

Microscopic observation of preserved samples confirmed that the cyanobacteria species that were positive in qPCR were present in the lake samples. Other species were also present such as *Planktolyngbya* spp., *Microcystis viridis*, and *Cylindrospermopsis curvispora*. There was notably less cyanobacteria and more diatoms in Belews Lake than in any other lake.

Toxin Gene Abundance

The microcystin *mcyH+/A*- gene was present in four of six lakes sampled and seasonal patterns were evident (Figure 16). *McyH+/A*- DNA abundances ranged from 0 – 642.2 ng $ml^{-1}$ in Randleman Reservoir to 0 - 1272.2 ng $ml^{-1}$ in Oak Hollow Lake (Table 10). Belews Lake and Lake Brandt had no positive samples for the *mcyH+/A*- gene. There were no significant differences in *mcyH+/A*- DNA abundance among the four lakes where it was present.

There were significant correlations between *mcyH+/A*- DNA abundance and individual taxon SSU rDNA abundance (Table 11). *Aphanizomenon/ Anabaena* spp. SSU rDNA abundance and *C. raciborskii* were both significantly correlated with *mcyH+/A*- DNA abundance in Lake Mackintosh ($r_s$ = 0.611; $r_s$ = 0.465) and Randleman Reservoir ($r_s$ = 0.474; $r_s$ = 0.543). Total cyanobacteria SSU rDNA was significantly correlated with *mcyH+/A*- DNA abundance ($r_s$ = 0.496) in Oak Hollow Lake. There were no significant correlations between temperature and *mcyH/A*- target DNA abundance.

Anatoxin *PKS* DNA was present in all lakes and the range spanned 5 orders of magnitude (Figure 17). Belews Lake had the lowest amount of anatoxin *PKS* DNA ranging from 0 - 47.9 ng $ml^{-1}$ and Oak Hollow Lake had the most anatoxin *PKS* DNA ranging from 0 – 20184.8 ng $ml^{-1}$ (Table 10). There were no significant differences between years or among lakes. Overall, anatoxin *PKS* DNA abundance correlated with all potential anatoxin producing taxa SSU rDNA (Table 12). However, these correlations varied among lakes (Table 13). Anatoxin *PKS* DNA abundance was significantly correlated with temperature in Lake Mackintosh ($r_s$= 0.641, p = 0.004, n = 18), and Oak Hollow Lake ($r_s$= 0.523, p = 0.043, n = 14) (Table 14).

The cylindrospermopsin *PKS* gene was not detected in any of the lakes. To confirm this result, an additional set of primers (MTF2 / MTR) specific to the *PS* gene were run on select samples which showed high concentrations of *C. raciborskii* DNA (Table 3). These samples were also negative.

ELISA Assay

Microcystins were present in selected samples from four of six lakes (Table 15). Microcystins were present in all 2011samples tested from City Lake, ranging from 0.19 – 0.32 ppb, but below the accurate reportable limit in all 2012 samples tested. Microcystins were within detectable levels in 5 of 7 samples tested from Randleman Reservoir ranging from 0.16 - 0.18 ppb, and in 1 of 5 samples from Lake Mackintosh (0.17 ppb). There were no correlations between microcystin levels, *mcyH+/A*- gene abundance, total cyanobacteria SSU rDNA, or taxon specific SSU rDNA. Samples were below the accurate reportable limit as indicated by the ELISA manufacturer (ppb < 0.16

ppb) in Lake Brandt and Belews Lake. All microcystin levels were below the acceptable guideline for microcystins in recreational water (20 ppb) and drinking water (1ppb).

Cylindrospermopsin abundance was below detectable limits (< 0.05 ppb) in all samples. This was expected since cylindrospermopsin *PKS or PS* genes were not found using qPCR.

# CHAPTER IV

# DISCUSSION

Cyanobacteria are an emerging environmental and health concern. Global warming and climate change are affecting the timing of lake stratification, precipitation, and drought patterns providing conditions for cyanobacteria proliferation (Paerl and Huisman 2010). Lake eutrophication, increased nutrients influx from urban areas, and decreased export of nutrients due to drought conditions also promote cyanobacteria blooms. Since many lakes are water supply reservoirs, increased populations of toxigenic cyanobacteria could pose health risks. Better analytical tools can help water quality managers and regulatory agencies assess and manage cyanobacteria and cyanotoxin abundance and develop better strategies to reduce environmental and health risk.

## Taxon Abundance Correlations

This study used qPCR to assess presence and relative abundance of five cyanobacteria taxa in 6 lakes. Results obtained using qPCR in these six NC lakes during the 2011-2012 sampling seasons were consistent with previous work using conventional methods to identify cyanobacteria taxa **(**Glasgow and Burkholder 2003, Touchette *et. al.* 2007, Stewart 2011**)**. Stewart (2011) found similar taxa and seasonal patterns using qPCR on City Lake water samples; although, he did not report the presence of *Microcystis aeruginosa* because he did not use primers for that species.

The correlation among cyanobacteria taxa in all lakes is as expected, and is likely due to similar environmental factors. In general, North Carolina reservoirs experience a moderate sedimentation rate and frequent reoccurring suspended sediment loading into reservoirs, making conditions conducive to cyanobacteria growth (Touchette *et. al.*2007). All of the lakes studied in this experiment are eutrophic reservoirs (NCDENR 2009, NCDENR 2012) with nutrient input from various sources, except for Belews Lake, which is categorized as oligotrophic (NCDENR 2010). All these lakes are located near urban areas with residential housing and commercial buildings, and near farmlands that contribute nutrients to the lakes, except for Belews Lake and Lake Mackintosh which are surrounded mostly by suburban housing and farmlands (NCDENR 2009). All of these lakes are water supply reservoirs with the exception of Belews Lake which was constructed to provide cooling water for a coal fired power plant.

Belews Lake and Lake Mackintosh appear to have lower abundances of taxon specific SSU rDNA and total cyanobacteria SSU rDNA. These lakes are the two largest lakes that were sampled and are both of moderate age. Randleman reservoir is also large, but it is also the youngest lake (2 yrs) and is still in an early ontogenic stage and thus may exhibit different characteristics than older lakes.

Lake Brandt, Oak Hollow Lake, and City Lake had year-to-year variation. These lakes are the smallest and shallowest lakes (Table 1). City Lake and Oak Hollow Lake are also both run-of-river impoundments with high flow during rain events and long residence time in periods of drought. Drought conditions in 2011 may have affected taxon abundances in these lakes. Ahn *et. al*. (2011) found that the most influential factor

in cyanobacteria growth was water temperature. Water temperatures were similar to temperatures suitable for cyanobacteria growth (Saker and Griffith 2010, Rapala *et. al.*1993., Yin *et. al.* 1997).

Toxin Gene Abundance

Toxin synthesis may not be correlated with taxon abundance for a number of reasons. First, potentially toxigenic species may not have toxigenic genes or may have variation within the toxin genes that affect toxin production (Figure 2). Second, multiple species often contribute to the production of a single toxin (Figure 18) and different species may contribute different amounts of a given toxin. If a toxin producing species was not measured in this study, correlations would be weak. Third, toxin production may depend on environmental cues that are different among taxa or in different lakes.

The cylindrospermopsin *PKS* gene was not found in any of the lakes studied even though multiple potential cylindrospermopsin producing species were found. This is consistent with results from other studies in NC lakes (Glasgow and Burkholder 2003, Touchette *et. al.* 2007, Stewart 2011). Other studies have not found cylindrospermopsin *PKS* and *PK*, or another gene in the cylindrospermopsin pathway, *CYN* (personal communication, B.Touchette, Elon University; E. Allen, NC State University; and C. Britton, Methodist University). Cylindrospermopsins were below detectable limits (ppb < 1 ppb) in NC Reservoirs (n = 9) in 2002 (Glasgow and Burkholder 2003). However, the *CYN* gene has been found in Currituck Sound (Calandrino 2009) and cylindrospermopsins were found in Knights of Columbus Pond, a freshwater pond located in Wake County, NC (Glasgow and Burkholder 2003).

Lack of correlation between taxon abundance and *mcyH+/A*- DNA abundance may be due to variation of gene order within the microcystin gene cassette (Figs.1, 2) which could prevent or alter qPCR amplification. The primers used in this study were designed to flank the transition region between *mcyH* and *mcyA* of *Planktothrix* spp.**(**Kurmayer *et al.* 2004), thus it may be generating different amplicons if the order of *mcy* genes in the *mcy* cassette differs among species within mixed environmental samples. Further, if there has been a *mcy* gene deletion event within a population and there are remnants of the genes the cyanobacteria would lose the ability to produce microcystins but the genes may still amplify using qPCR. Melt curves analysis revealed two obvious peaks supporting the idea that two amplicons may have been produced. The *M. aeruginosa* primers used in this study measure all *M. aeruginosa* SSU rDNA present within collected samples. However, some of these cyanobacteria may not be toxigenic. The *mcyH+/A*- primer pair would only amplify toxigenic strains leading to lack of correlations between taxa specific SSU rDNA abundances and *mcyH/A* gene abundances. Primers designed to *mcyD* within the *mcy* cassette may be useful in future studies if microcystin production is linked predominantly to *Microcystis aeruginosa* within a given lake. Davis *et.al.* (2009) used primers designed to the *mcyD* gene, which is found only in toxigenic *Microcystis aeruginosa* and was significantly correlated with microcystin levels.

Multiple species may contribute to the production of a single toxin (Figure 18). *Microcystis, Planktothrix*, *Oscillatoria*, *Nostoc*, *Anabaena*, and *Anabaenopsis* and *Hapalosiphon* spp. all have the potential to produce at least one microcystin congener

(WHO 2003). *Anabaena, Aphanizomenon, Oscillatoria* and *Cylindrospermum* spp. all have the ability to produce anatoxins. *Cylindrospermopsis raciborskii* is thought to be the dominant producer of cylindrospermopsin. This study only measured *Microcystis aeruginosa* SSU rDNA abundance and *Aphanizomenon/ Anabaena* spp. SSU rDNA abundance and *Cylindrospermopsis raciborskii* SSU rDNA from this list of toxin producers and thus other species may be contributing to the cumulative *mcyH+/A*- DNA abundance and anatoxin *PKS* abundances. In fact, *Planktothrix agardhii* contains approximately double the amount of microcystin content per cell than *Microcystis aeruginosa* and could pose a threat with lower abundances than *Microcystis aeruginosa.*

Environmental cues may trigger toxin production. For example, light availability may play a role in whether these toxin genes are transcribed in *Microcystis aeruginosa* (Kaebernick *et. al.* 2001). Water temperatures have been previously found to affect anatoxin production. *Aphanizomenon flos-aquae*. *Aphanizomenon flos-aquae* (strains 3, 14, 167) and *Anabaena mendotae* (strain 130) all produced more toxin between 28$^{o}$C and 32$^{o}$C (Rapala *et.al.*1993). Water temperatures were correlated with anatoxin *PKS* gene abundance in Lake Mackintosh, but not in other lakes where temperature data were available.

In some lakes there was a strong correlation between anatoxin *PKS* DNA abundance and *Aphanizomenon/ Anabaena* spp. SSU rDNA abundance. The correlation between anatoxin *PKS* gene and *Aphanizomenon/ Anabaena* spp. SSU rDNA abundance is expected due to high primer specificity to *Aphanizomenon* spp. when these primers were designed. Anatoxin ELISA assays became commercially available in 2012

(Abraxis, PN 520051**)**. Use of this ELISA assay could reveal a correlation between anatoxin levels, *Aphanizomenon/ Anabaena* spp. SSU rDNA, and anatoxin *PKS* DNA abundance.

Utility of qPCR

The utility of qPCR to quantify cyanobacteria and cyanotoxin gene abundance can be enhanced. As sequence data for cyanobacteria species increases, more specific primers can be developed along with improved reference standards (positive controls). For example, in this study the primers used to amplify *Aphanizomenon issatschenkoi* also amplified known *Anabaena* spp. standards. When the primers to *Aphanizomenon issatschenkoi* (designated OTU8 in Stewart's [2008] study) were designed its 450 bp amplicon was a >98.7% match to *Aphanizomenon issatschenkoi* in Genbank (accession # EU078536.1). These two genera are easy to distinguish morphologically but they are genetically similar. Lyra *et.al*. (2001) found a 99.9% similarity between a toxigenic *Anabaena* spp. and non-toxigenic *Aphanizomenon* spp. 16sRNA. Further, questions about primer design and specificity arose when a *Planktolyngbya* species was observed in samples in this study even though the presence of *Lyngbya wollei* was not apparent (*L. wollei* standards were run with these primers as a positive control). When primers were compared to Genebank *Lyngbya wollei, Plectonema wollei, Aerosakkonema funiforme* (*Oscillatoriales*) and *Oscillatoria sancta*, all showed 100% query coverage and identity indicating that there may be amplification from multiple closely related taxa.

Additionally, qPCR could also be improved by gaining knowledge of species specific gene copy number for target genes. This will allow for better comparisons of organisms abundance among lakes and across species.

qPCR targeting cyanotoxin genes could be beneficial in predicting emerging cyanobacteria water quality problems. The presence and abundance of toxin genes could give water quality managers insight into development of water management strategies to assess health risk. For example, this experiment revealed that there was no cylindrospermopsin *PKS* DNA and no detectable amount of cylindrospermopsins in any of the six lakes and no *mcyH+/A*- gene presence in Belews Lake or Lake Brandt. Thus, cyanotoxin health risk appears low in these instances. Conversely, anatoxin may be an emerging problem since the anatoxin *PKS* gene were present in all of the lakes sampled. Lack of clear correlation between potentially toxic cyanobacteria SSU rDNA, *mcyH+/A*- gene, and microcystin abundance supports the continued use of microcystin ELISAs to assess public health risk until other methods are further developed. For example, reverse transcriptase PCR could potentially be used to assess toxin production by monitoring the amount of gene transcript within environmental samples. Even though our ELISA assay revealed low levels of microcystins in the water, the presence of the toxin gene reveals that this could become an emerging and acute problem if conditions become more conducive to cyanobacteria growth and blooms.

Long term value could be gained from developments in qPCR for the identification of cyanobacteria. Species specific primers used in conjunction with qPCR could be used for a quick way to identify and quantify possibly toxigenic cyanobacteria

species within lakes as well as their seasonal abundance patterns. It may even eliminate the need for visual identification.

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# APPENDIX A

# TABLES AND FIGURES

**Table 1. Piedmont North Carolina Lakes Sampled in This Study**.

| | Year Built | Size (Acres) | Mean Depth(m) | Volume ($10^6 m^3$) | Purpose |
|---|---|---|---|---|---|
| City Lake | 1928 | 340 | 4.8 | 4.8 | Water supply |
| Oak Hollow Lake | 1972 | ~800 | 6.4 | 11 | Water supply |
| Randleman Reservoir | 2010 | ~3000 | ? | ? | Water supply |
| Lake Brandt | 1925 | 816 | 2.3 | 84 | Water supply |
| Lake Mackintosh | 1991 | 1100 | 18 | 29 | Water supply |
| Belews Lake | 1973 | 3863 | 14.9 | 228 | Power plant cooling |

**Table 2. Taxon Specific SSU rDNA Primers Used in This Study.**

| Organism | Primer Name | Primer Sequence 5' – 3' | Source |
|---|---|---|---|
| *Aphanizomenon/ Anabaena spp.* | F59-08_57 | GTGGCTAATACCGAATGTGCCGA | Stewart 2011 |
| | R409-57_08 | CCCTTWACGCCCAATCATTCCGGATAA | |
| *Cylindrospermopsis raciborskii* | 102F | GGTGAAAGATTTATCGCCTGGAGATGA | Stewart 2011 |
| | CYA 781(a/b) | GACTAC(T/A)GGGGTATCTAATCCC(A/T)TT | Nubel *et al.* 1997 |
| *Lyngbya wollei* | LwoolF | GAT TAA TTG CCA GAA GAT GAG C | Stewart 2011 |
| | CYA 781(a/b) | GACTAC(T/A)GGGGTATCTAATCCC(A/T)TT | Nubel *et al.* 1997 |
| *Microcystis aerugenosa* | Micro233F | HCTAATTGGCCTGRAGAAGAGC | Tomioka *et. al.* 2011 |
| | CYA 781(a/b) | GACTAC(T/A)GGGGTATCTAATCCC(A/T)TT | Nubel *et al.* 1997 |
| General Cyanobacteria | CYA 106(f) | CGGACGGGTGAGTAACGCGTGA | Nubel *et al.* 1997 |
| | CYA 781(a/b) | GACTAC(T/A)GGGGTATCTAATCCC(A/T)TT | |

**Table 3. Cyanotoxin Gene Primer Used in This Study.**

| Toxin | Primer Name | Primer Sequence 5' - 3' |
|---|---|---|
| Cylindrospermopsin *PKS* | M13 | GGCAAATTGTGATAGCCACGAGC |
| (Schembri *et al*. 2001) | M14 | GATGGAACATCGCTCACTGGTG |
| Cylindrospermopsin *PS* | MTF2 | GCNGG(CT)GG(CT)GCNTA(CT)GTNCC |
| (Schembri *et al*. 2001) | MTR | CCNCG(AGT)AT(TC)TTNAC(TC)TG |
| Microcystin *mcyH/A* | McyH+ | GGTCGATTAATTCGGCCTTCC |
| (Kurmayer *et al.* 2004) | McyA- | AAACGGAACTATCGGTTGCCTC |
| Anatoxin *PKS* | atxaf | TCGGAAGCGCGATCGCAAATCG |
| (Ballot *et al.* 2010) | atxar | GCTTCCTGAGAAAGGTCCGCTAG |

**Table 4. Mean and Range of SSU rDNA (pg ml$^{-1}$) in Study Lakes.** Ranges are reported with outliers removed; however, outliers are given below the range. DNA abundances are comparable among lakes within taxon but not among lakes between taxa due to different reference DNA during qPCR.

| | *Aphanizomenon/ Anabaena spp.* | *Cylindrospermopsis raciborskii* | *Lyngbya wollei* | *Microcystis aeruginosa* | Total Cyanobacteria |
|---|---|---|---|---|---|
| City Lake | 4<br>0.0 – 24.6 | 0.4<br>0.0 – 2.8 | 262<br>1.2 – 1063.0 | 330.6<br>0.010 – 1827.6 | 17.6<br>0.003 – 139.6 |
| Oak Hollow Lake | 5<br>0.0 – 15.8 | 1.5<br>0.0 – 11.2 | 595.6<br>0.0 – 2016.7<br>3404.8 | 1242<br>0.094 – 5288.5 | 48.341<br>0.00 – 181.8 |
| Randleman Reservoir | 8.7<br>0.1 – 38.2 | 1.1<br>0.0 – 11.8 | 101.4<br>0.0 – 345.6 | 2600.4<br>12.4 – 24262.2 | 8.2<br>0.00 – 30.4 |
| Lake Brandt | 3.3<br>0.0 – 16.901 | 0.3<br>0.0 – 1.9 | 628.8<br>0.0 – 1970.6 | 178.2<br>0.5 – 1034.2<br>173583.3 | 22.5<br>0.00 – 188.6<br>405.5 |
| Lake Mackintosh | 4.1<br>0.1 – 19.0<br>47.1 | 2<br>0.00 – 8.2<br>16.6 | 56.6<br>0.2 – 210.1 | 76.7<br>0.0 – 646.6 | 22.9<br>0.00 – 395.5 |
| Belews Lake | 0.04<br>0.0006 – 0.270 | 0.0005<br>0.00 – 0.0036 | 33.5<br>0.0 – 84.0 | 11.7<br>0.0 – 38.2 | 1<br>0.00004 – 6.3 |

**Table 5. Significant Taxon Specific SSU rDNA Comparisons among Lakes.** n = 18.

| Taxon specific SSU rDNA | Pairwise Compared Lakes | | | p value |
|---|---|---|---|---|
| *Aphanizomenon/ Anabaena* spp. | Belews Lake | - | City Lake | 0.005 |
| | Belews Lake | - | Oak Hollow Lake | < 0.0001 |
| | Belews Lake | - | Randleman Reservoir | < 0.0001 |
| | Belews Lake | - | Lake Brandt | 0.002 |
| | Belews Lake | - | Mackintosh | < 0.0001 |
| *Cylindrospermopsis raciborskii* | Belews Lake | - | City Lake | 0.028 |
| | Belews Lake | - | Oak Hollow Lake | <0.0001 |
| | Belews Lake | - | Randleman Reservoir | 0.001 |
| | Belews Lake | - | Mackintosh | < 0.005 |
| *Lyngbya wollei* | Belews Lake | - | Oak Hollow Lake | 0 .001 |
| | Belews Lake | - | Lake Brandt | <0.0001 |
| | Lake Mackintosh | - | Oak Hollow Lake | 0.008 |
| | Lake Mackintosh | - | Lake Brandt | 0.001 |
| *Microcystis aeruginosa* | Belews Lake | - | Oak Hollow Lake | <0.0001 |
| | Belews Lake | - | Randleman Reservoir | <0.0001 |
| | Lake Mackintosh | - | Oak Hollow Lake | 0.012 |
| | Lake Mackintosh | - | Randleman Reservoir | <0.0001 |

**Table 6. Significant Taxon Specific SSU rDNA Differences between Years within Lakes using Wilcoxon Mann Whitney.**

| Lake | Taxon specific SSU rDNA 2011 compared to 2012 | p- value |
|---|---|---|
| City Lake | *L. wollei* | p = 0.001 |
| Oak Hollow Lake | *Aphanizomenon/ Anabaena spp.* | p = 0.035 |
| | *L. wollei* | p = 0.043 |
| | Total Cyanobacteria | p = 0.001 |
| Lake Brandt | *Aphanizomenon/ Anabaena spp.* | p = 0.028 |

**Table 7. Spearman's Rank Correlations of Taxa and Water Temperature**

| | *Aphanizomenon/ Anabaena spp.* | *C. raciborskii* | *Lyngbya wollei* | *M. aeruginosa* | Total cyanobacteria |
|---|---|---|---|---|---|
| City Lake | $r_s = 0.46$ | $r_s = 0.27$ | $r_s = 0.631^{**}$ | $r_s = 0.45$ | $r_s = 0.36$ |
| Oak Hollow Lake | $r_s = 0.23$ | $r_s = 0.38$ | $r_s = 0.699^{**}$ | $r_s = 0.41$ | $r_s = 0.36$ |
| Lake Mackintosh | $r_s = 0.691^{**}$ | $r_s = 0.657^{**}$ | $r_s = 0.38$ | $r_s = -0.24$ | $r_s = 0.46$ |

**$p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 8. Spearman's Rank Correlations among Taxa and Total Cyanobacteria SSU rDNA (All Lakes Combined)**

| | *Aphanizomenon/ Anabaena spp.* | *Cylindrospermopsis raciborskii* | *L. wollei* | *Microcystis aeruginosa* |
|---|---|---|---|---|
| *Cylindrospermopsis raciborskii* | $r_s = 0.636^{**}$ | | | |
| *L. woolei* | $r_s = 0.473^{**}$ | $r_s = 0.326^{**}$ | | |
| *Microcystis aeruginosa* | $r_s = 0.485^{**}$ | $r_s = 0.330^{**}$ | $r_s = 0.372^{**}$ | |
| Total Cyanobacteria | $r_s = 0.093$ | $r_s = 0.116$ | $r_s = 0.175$ | $r_s = -0.053$ |

** $p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 9. Spearman's Rank Correlations among Taxa within Study Lakes.**

| | | *Aphanizomenon/ Anabaena* spp. | *C. raciborskii* | *L. wollei* | *M. aeruginosa* |
|---|---|---|---|---|---|
| City Lake | *C. raciborskii* | 0.617** | - | - | - |
| | *L. wollei* | 0.44 | 0.43 | - | - |
| | *M. aeruginosa* | 0.34 | 0.15 | 0.35 | - |
| | Total cyanobacteria | 0.06 | 0.08 | 0.565* | .547* |
| Oak Hollow Lake | *C. raciborskii* | 0.620** | - | - | - |
| | *L. wollei* | 0.44 | 0.663** | - | - |
| | *M. aeruginosa* | 0.27 | 0.33 | 0.23 | - |
| | Total cyanobacteria | 0.675** | 0.605** | 0.565* | 0.22 |
| Randleman Reservoir | *C. raciborskii* | 0.41 | - | - | - |
| | *L. wollei* | 0.889** | 0.3 | - | - |
| | *M. aeruginosa* | 0.579** | 0.06 | 0.32 | - |
| | Total cyanobacteria | 0.475* | 0.14 | 0.545* | 0.17 |
| Lake Brandt | *C. raciborskii* | 0.42 | - | - | - |
| | *L. wollei* | 0.39 | 0.12 | - | - |
| | *M. aeruginosa* | 0.23 | 0.39 | -0.02 | - |
| | Total cyanobacteria | 0.21 | 0.22 | 0.44 | -0.01 |
| Lake Mackintosh | *C. raciborskii* | 0.528* | - | - | - |
| | *L. wollei* | 0.740** | 0.38 | - | - |
| | *M. aeruginosa* | -0.02 | -0.04 | -0.15 | - |
| | Total cyanobacteria | 0.538* | 0.559* | 0.504* | -0.21 |
| Belews Lake | *C. raciborskii* | 0.19 | - | - | - |
| | *L. wollei* | 0.3 | 0.14 | - | - |
| | *M. aeruginosa* | 0.29 | -0.05 | 0.36 | - |
| | Total cyanobacteria | 0.502* | 0.21 | 0.35 | 0.04 |

** $p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 10. Mean and Range of Toxin Gene DNA (ng ml$^{-1}$) in Study Lakes.** Outliers have been removed for range analysis; however, outliers are given after the range because they might be representative of blooms. DNA abundances are comparable among lakes within taxa but not among lakes between taxa due to different DNA reference standards used for qPCR. n = 18 for lake samples with *mcyH+/A*- DNA, n = 19 for lake samples with anatoxin *PKS* DNA.

| | *mcyH+/A*- | Anatoxin *PKS* |
|---|---|---|
| City Lake | 198.9<br>0.1 – 801.6 | 1348.5<br>0.0 – 12133.4 |
| Oak Hollow Lake | 191.6<br>0.5 – 1272.2 | 3145.9<br>0.0 – 20184.8<br>23249.6 |
| Randleman Reservoir | 305.2<br>0.0 – 776.2 | 1231.6<br>0.0 – 11902.8 |
| Lake Brandt | | 200.3<br>0.0 – 1419.0 |
| Lake Mackintosh | 117.1<br>0.0 – 642.2<br>1347.1 | 517.4<br>0.0 – 2839.2 |
| Belews Lake | | 5.4<br>0.0 – 47.9 |

**Table 11. Spearman's Rank Correlations of Taxa and *Mcy H+/A-* DNA.** n = 18.

| | *Aphanizomenon/ Anabaena* spp. | *C. raciborskii* | *M. aeruginosa* | *Aphanizomenon/ Anabaena* spp. + *C. raciborskii* | *Aphanizomenon/ Anabaena* spp. + *M. aeruginosa* | *Aphanizomenon/ Anabaena* spp. + *C. raciborskii* + *M. aeruginosa* | Total Cyanobacteria |
|---|---|---|---|---|---|---|---|
| City Lake | $r_s = 0.168$ | $r_s = -0.283$ | $r_s = 0.209$ | $r_s = 0.147$ | $r_s = 0.172$ | $r_s = 0.172$ | $r_s = 0.147$ |
| Oak Hollow Lake | $r_s = 0.125$ | $r_s = 0.208$ | $r_s = 0.265$ | $r_s = 0.146$ | $r_s = 0.263$ | $r_s = 0.263$ | $r_s = 0.496^{*}$ |
| Randleman Reservoir | $r_s = 0.474^{*}$ | $r_s = 0.543^{*}$ | $r_s = 0.232$ | $r_s = 0.497^{*}$ | $r_s = 0.257$ | $r_s = 0.257$ | $r_s = -0.143$ |
| Lake Mackintosh | $r_s = 0.611^{**}$ | $r_s = 0.465^{*}$ | $r_s = 0.141$ | $r_s = 0.643^{**}$ | $r_s = 0.139$ | $r_s = 0.185$ | $r_s = 0.288$ |

** $p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 12. Spearman's Rank Correlations of Taxa and Cyanotoxin DNA.**
n = 74 for lake samples with *mcyH+/A*- DNA, n = 110 for lake samples with anatoxin *PKS* DNA.

| | Anatoxin *PKS* | *mcy*H+/A- |
|---|---|---|
| *Aphanizomenon/ Anabaena spp.* | $r_s = 0.432^{**}$ | $r_s = 0.185$ |
| *Cylindrospermopsis raciborskii* | $r_s = 0.411^{**}$ | |
| *Microcystis aeruginosa* | $r_s = 0.214^{*}$ | $r_s = -0.156$ |
| Total Cyanobacteria | $r_s = 0.160$ | $r_s = -0.021$ |

** $p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 13. Spearman's Rank Correlations of Taxa and Anatoxin *PKS* DNA.** n = 19.

| | *Aphanizomenon/ Anabaena* spp. | *Microcystis aeruginosa* | *Aphanizomenon/ Anabaena* spp. + *Microcystis aeruginosa* | Total Cyanobacteria |
|---|---|---|---|---|
| City Lake | $r_s$ = 0.368 | $r_s$ = 0.396 | $r_s$ = 0.477* | $r_s$ = 0.285 |
| Oak Hollow Lake | $r_s$ = 0.594** | $r_s$ = 0.495* | $r_s$ = 0.509* | $r_s$ = 0.677** |
| Randleman Reservoir | $r_s$ = -0.102 | $r_s$ = -0.383 | $r_s$ = -0.369 | $r_s$ = 0.293 |
| Lake Brandt | $r_s$ = 0.249 | $r_s$ = -0.282 | $r_s$ = -0.252 | $r_s$ = 0.530* |
| Lake Mackintosh | $r_s$ = 0.726** | $r_s$ = -0.425 | $r_s$ = -0.324 | $r_s$ = 0.740** |
| Belews Lake | $r_s$ = 0.233 | $r_s$ = -0.523* | $r_s$ = -0.516 | $r_s$ = 0.468* |

** $p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 14. Spearman's Rank Correlations of Cyanotoxins and Water Temperature.**

| | *mcyH+/A-* | Anatoxin *PKS* |
|---|---|---|
| City Lake | $r_s = 0.267$ | $r_s = 0.160$ |
| Oak Hollow Lake | $r_s = 0.353$ | $r_s = 0.523$* |
| Lake Mackintosh | $r_s = 0.428$ | $r_s = 0.641$** |

** $p \leq 0.01$ (2-tailed test)
* $p \leq 0.05$ (2-tailed test)

**Table 15. Microcystin (ppb) in Selected Water Samples.**

| | Date | Microcystins (ppb) |
|---|---|---|
| City Lake | 6/6/11 | 0.19 |
| | 7/5/11 | 0.31 |
| | 8/15/11 | 0.19 |
| | 9/12/11 | 0.19 |
| | 6/1/12 | <0.16 |
| | 7/11/12 | <0.16 |
| Oak Hollow Lake | 6/20/11 | 0.26 |
| | 8/14/11 | 0.19 |
| | 6/14/12 | <0.16 |
| | 7/11/12 | 0.17 |
| Randleman Reservoir | 8/3/11 | 0.16 |
| | 7/10/11 | 0.18 |
| | 8/17/11 | <0.16 |
| | 9/14/11 | <0.16 |
| | 1/11/12 | 0.17 |
| | 6/21/12 | <0.16 |
| | 8/31/12 | 0.17 |
| Lake Brandt | 9/16/11 | <0.16 |
| | 8/15/12 | <0.16 |
| Lake Mackintosh | 6/16/11 | 0.17 |
| | 6/30/11 | <0.16 |
| | 7/14/11 | <0.16 |
| | 7/28/11 | <0.16 |
| | 7/5/12 | <0.16 |
| Belews Lake | 7/12/11 | <0.16 |
| | 5/22/12 | <0.16 |
| | (-) | 0.0841 |
| | (-) | 0.0841 |

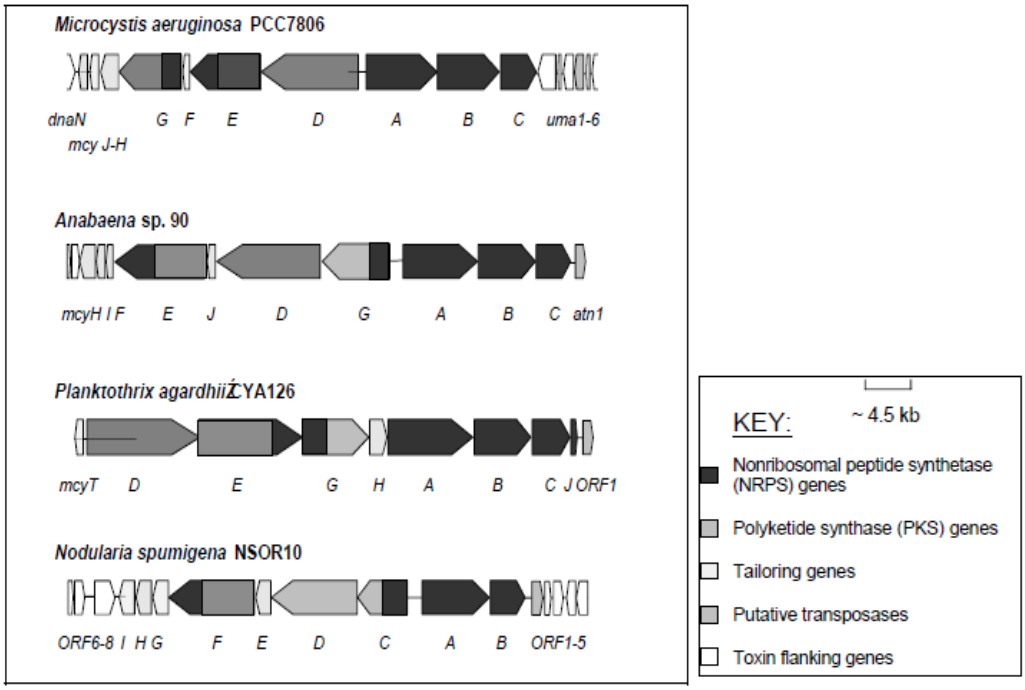


**Figure 1. Variations in *Mcy* Gene Order (from Haven 2008).**

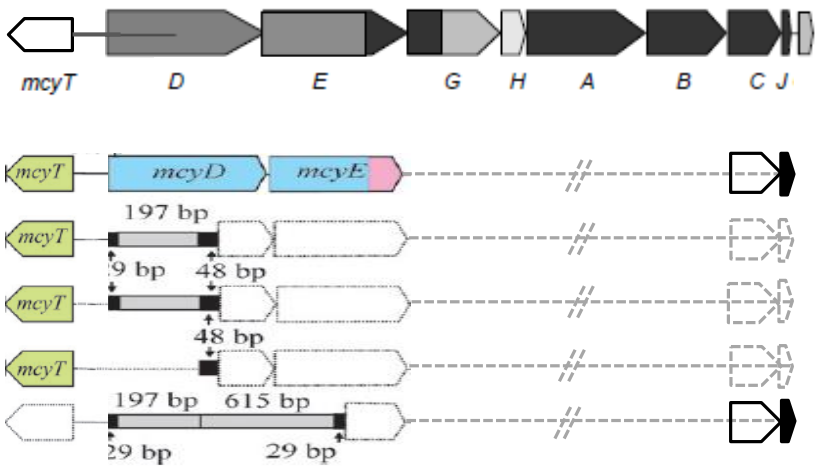


**Figure 2. Variations within the *Mcy* Gene Cassette when Deletion Events Occur and Gene Remnants Remain in *Planktothrix* spp. (modified from Christiansen *et. al.* 2008 and Haven 2008)**

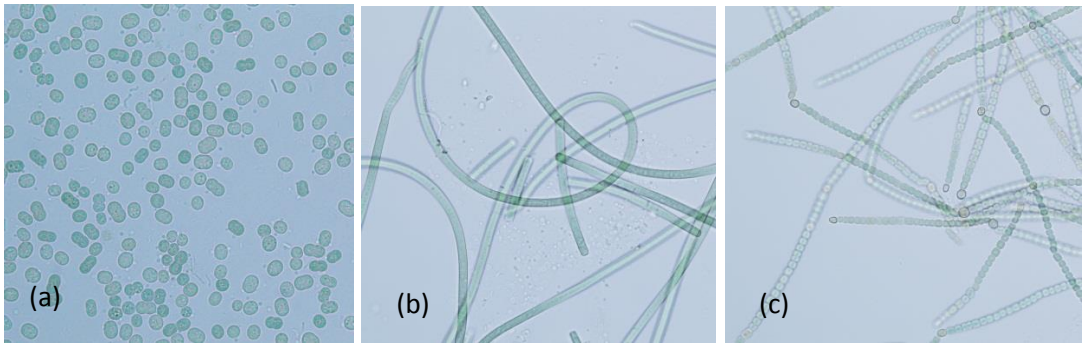


**Figure 3. Photographs of the Organisms used for *Microcystis aeruginosa* (a), *Lyngbya wollei* (b) and *Anabaena* spp.(c) Standards.**

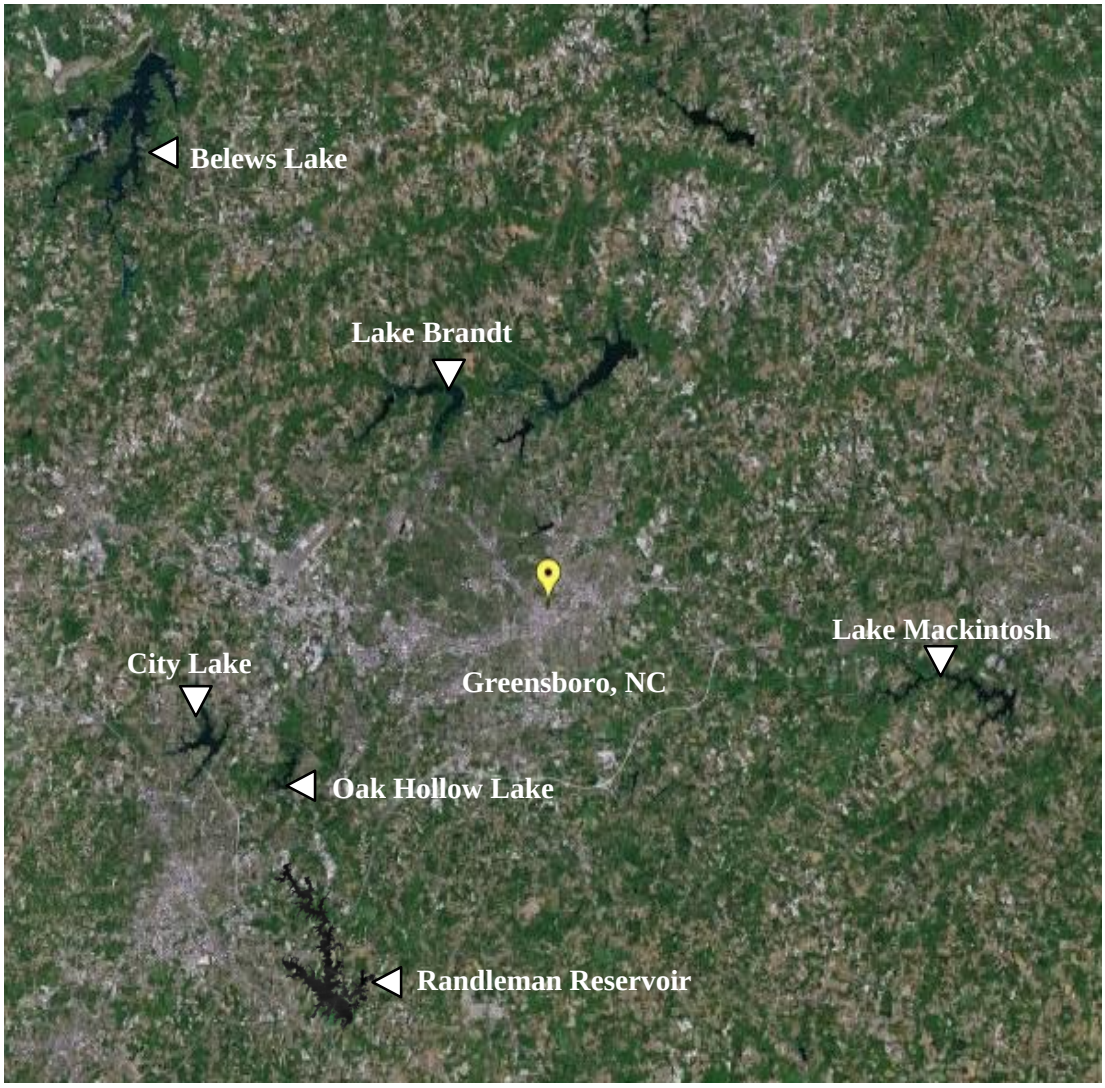


**Figure 4. Location of Six Study Lakes in the Piedmont of North Carolina**

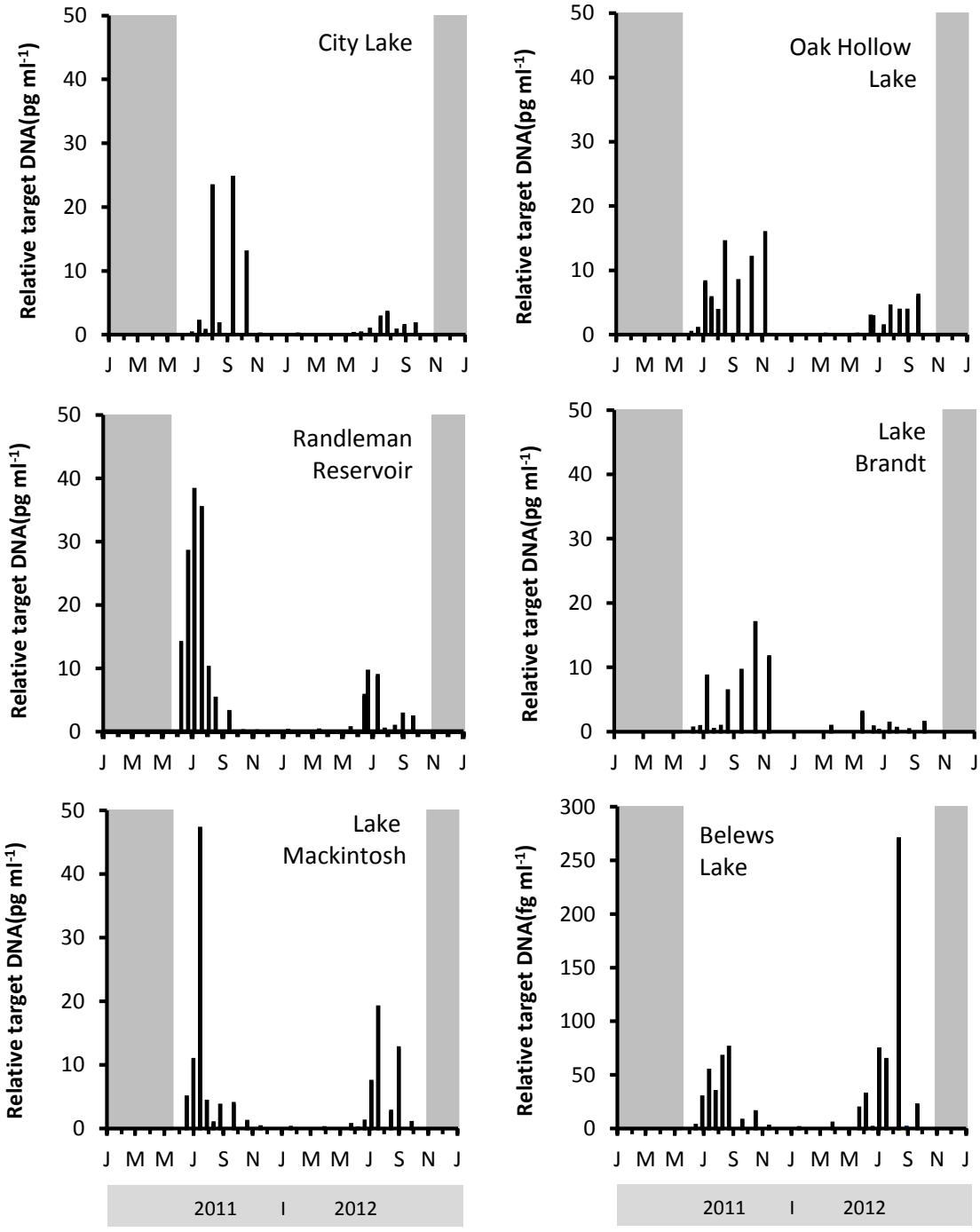


**Figure 5.** ***Aphanizomenon/ Anabaena*** **spp. SSU rDNA Abundance for Samples Taken between June 2011 and September 2012.**

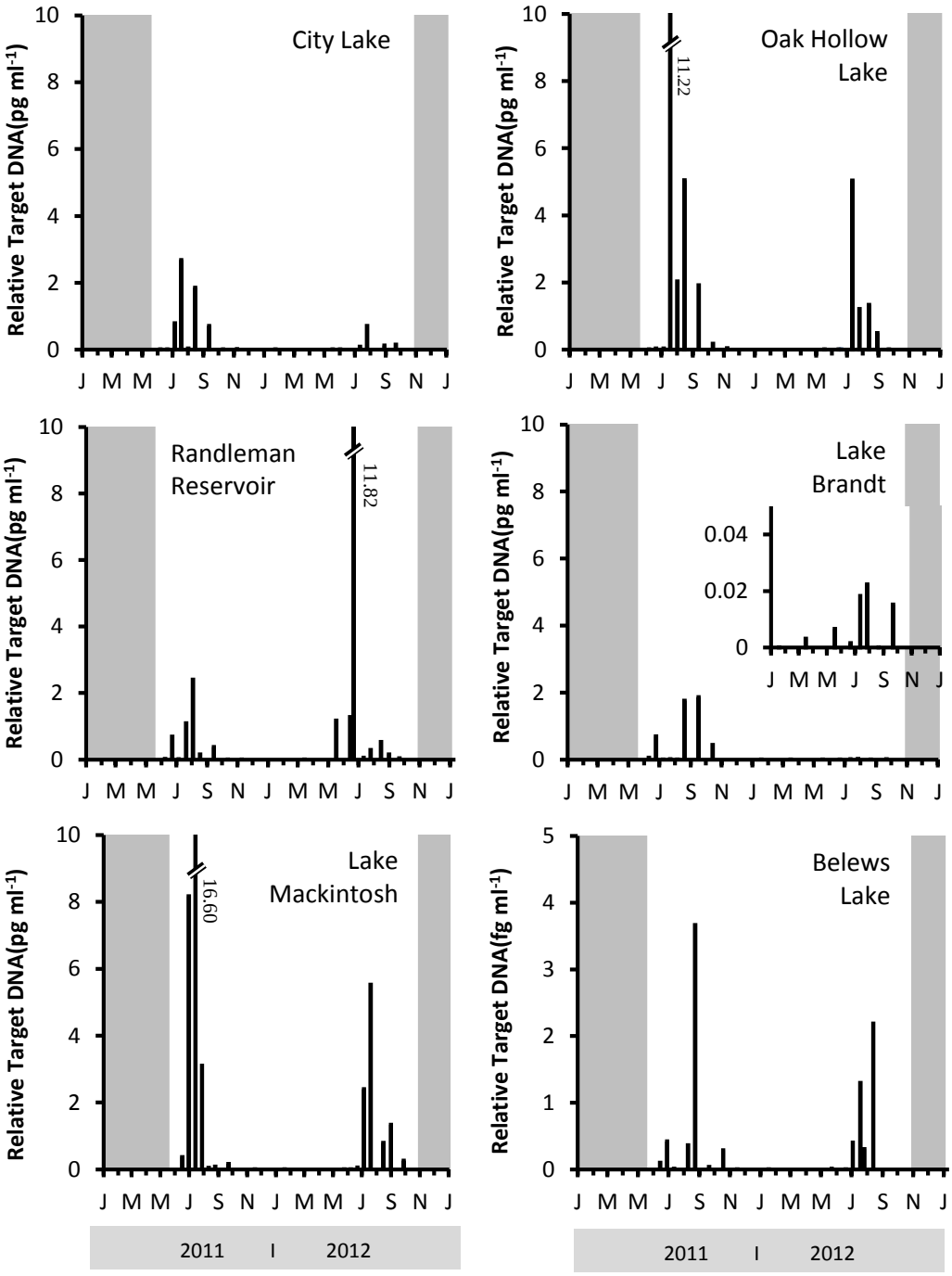


**Figure 6.** ***Cylindrospermopsis raciborskii*** **SSU rDNA Abundance for Samples Taken between June 2011 and September 2012.**

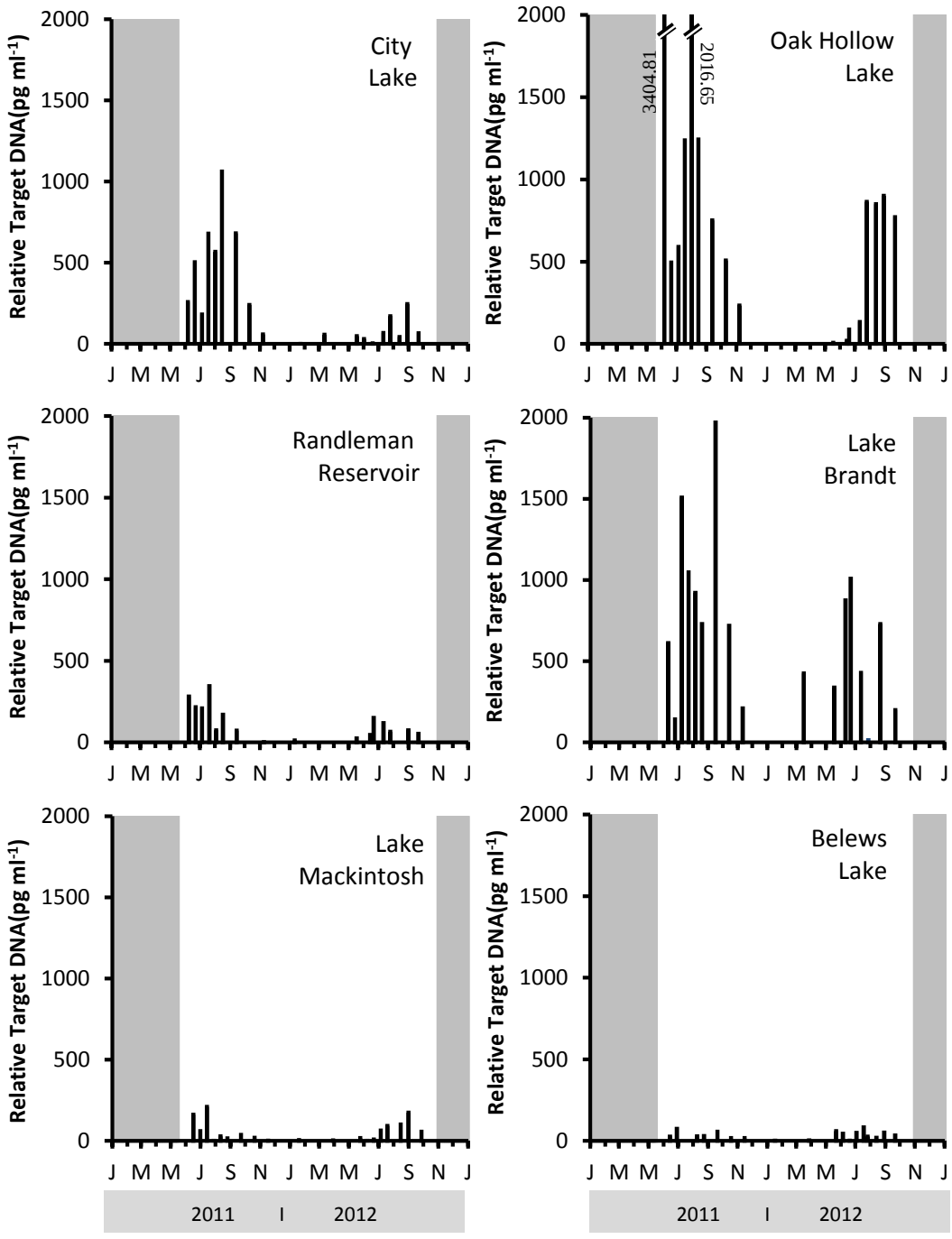


**Figure 7.** ***Lyngbya wollei*** **SSU rDNA Abundance for Samples Taken between June 2011 and September 2012.**

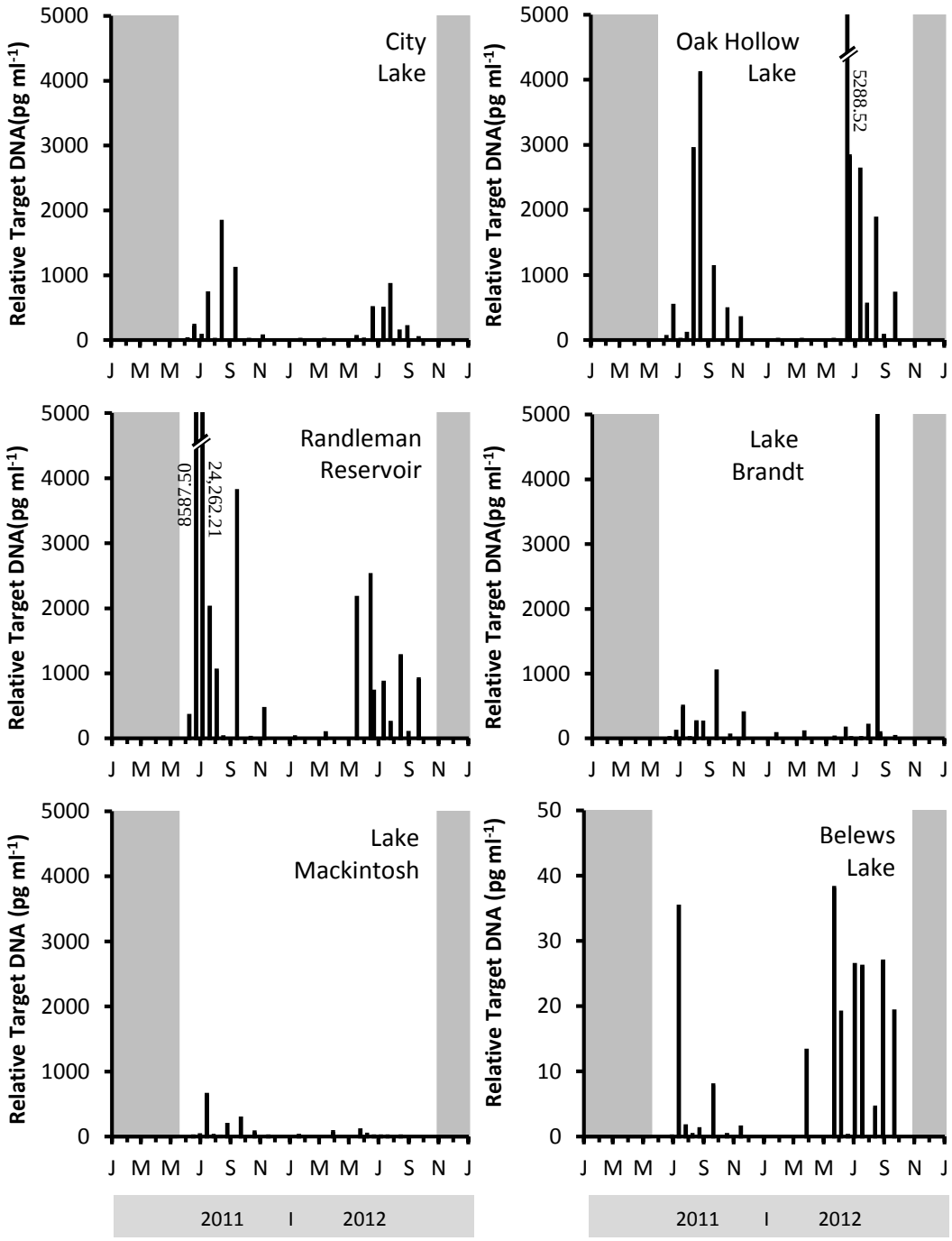


**Figure 8.** ***Microcystis aeruginosa*** **SSU rDNA Abundance for Samples Taken between June 2011 and September 2012.**

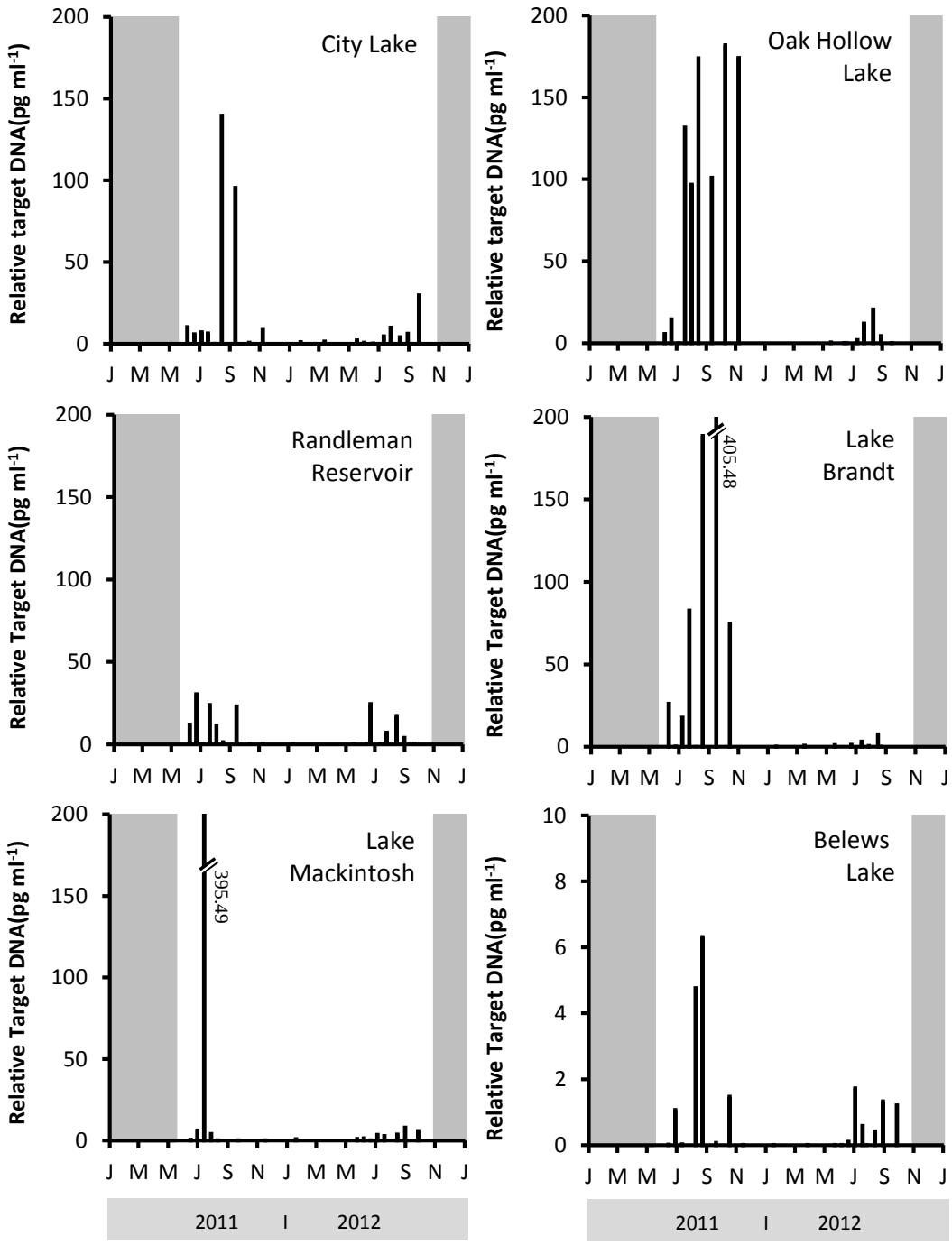


**Figure 9. Total Cyanobacteria SSU rDNA Abundance for Samples Taken between June 2011 and September 2012.**

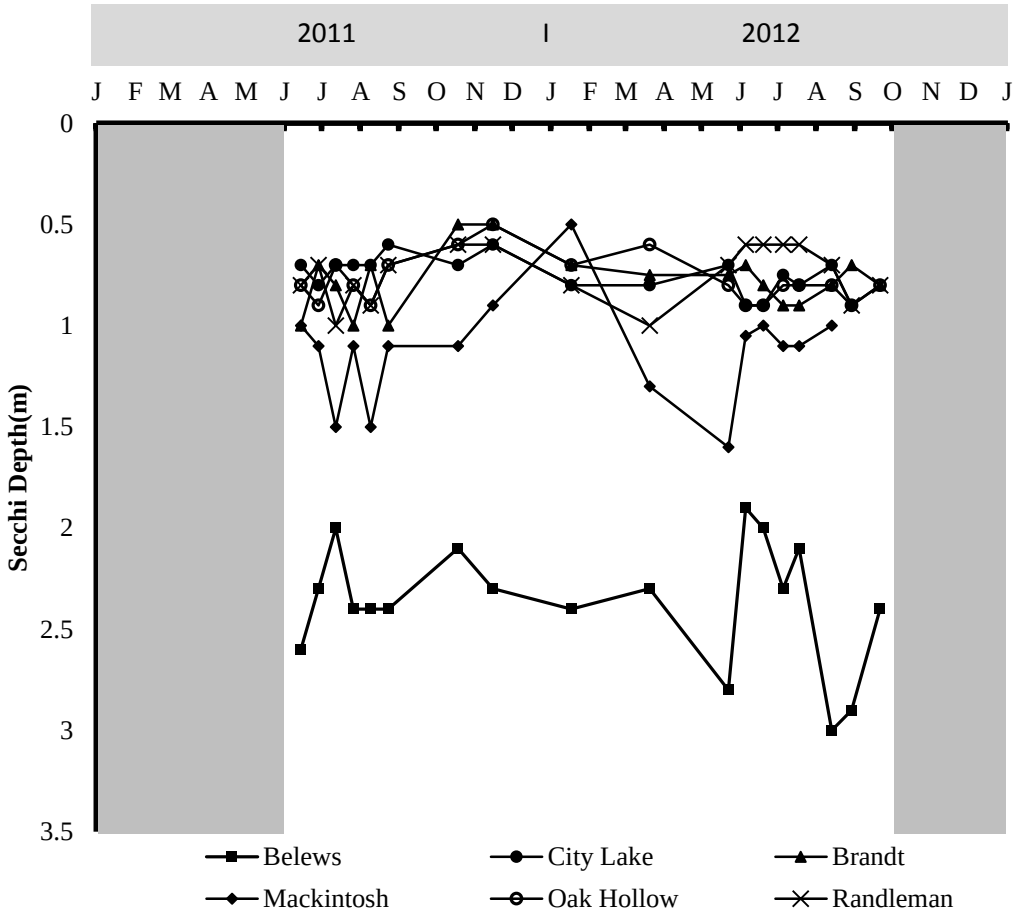


**Figure 10. Secchi Depths in Study Lakes from June 2011 - October 2012.**

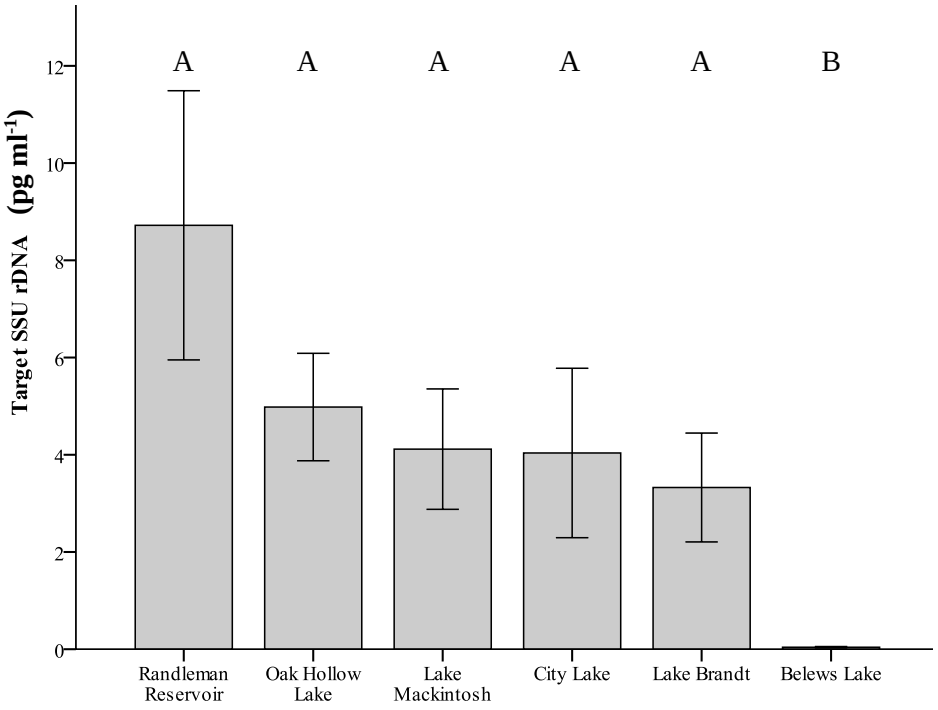


**Figure 11. Mean *Aphanizomenon*/ *Anabaena* spp. Target SSU rDNA ± SE.** Similar letters indicate lakes that show no significant differences in mean *Aphanizomenon*/ *Anabaena* spp. target SSU rDNA based on the nonparametric Kruskal-Wallis analysis.

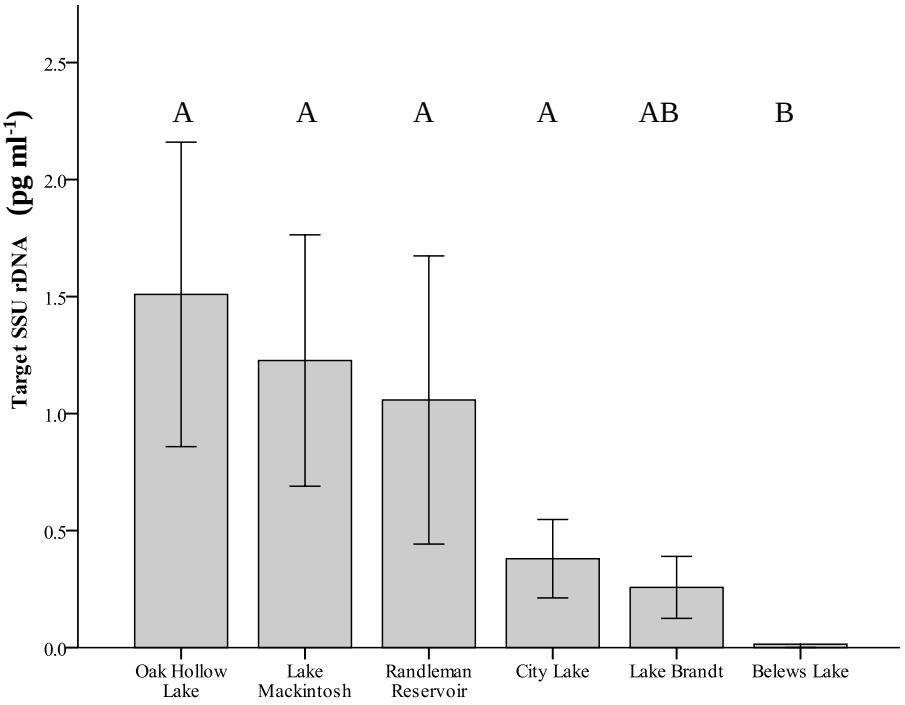


**Figure 12. Mean *Cylindrospermopsis raciborskii* Target SSU rDNA ± SE.** Similar letters indicate lakes that show no significant differences in mean *Cylindrospermopsis raciborskii* target SSU rDNA based on the nonparametric Kruskal-Wallis analysis.

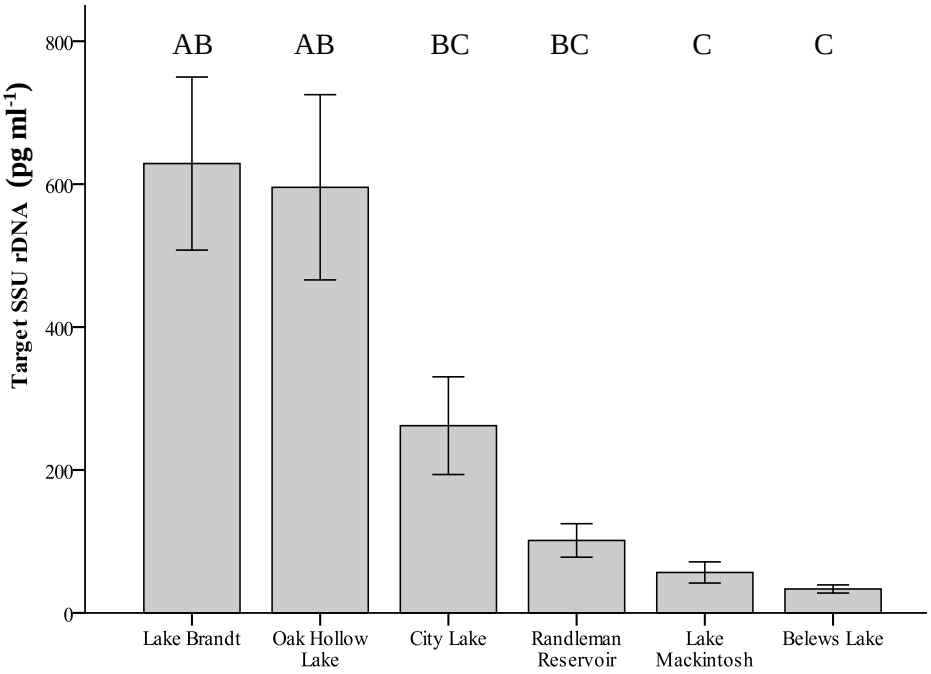


**Figure 13. Mean *Lyngbya wollei* Target SSU rDNA ± SE.** Similar letters indicate lakes that show no significant differences in mean *Lyngbya wollei* target SSU rDNA based on the nonparametric Kruskal-Wallis analysis.

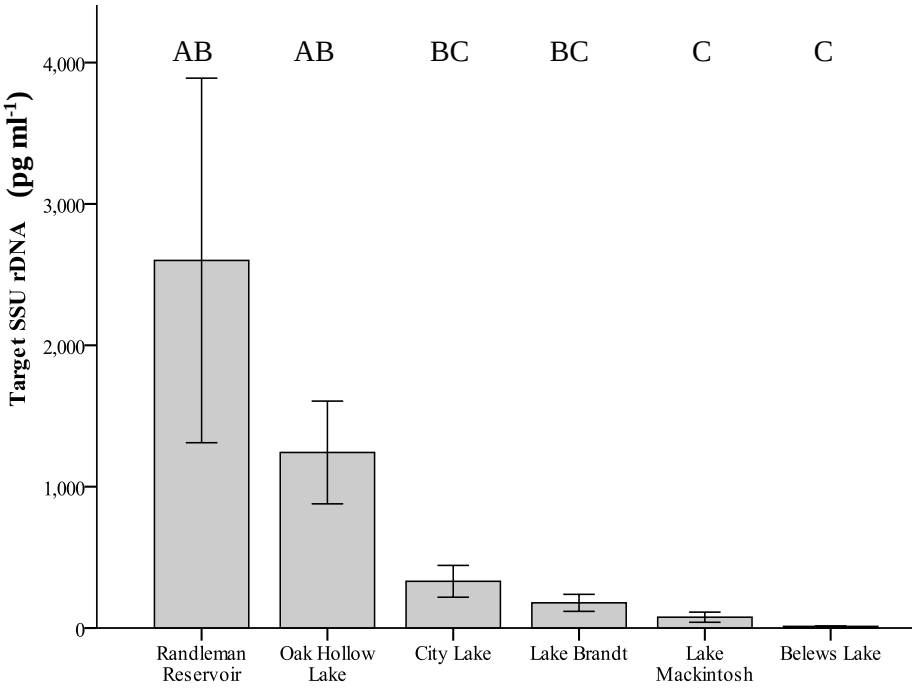


**Figure 14. Mean *Microcystis aeruginosa* Target SSU rDNA ± SE.** Similar letters indicate lakes that show no significant differences in mean *Microcystis aeruginosa* target SSU rDNA based on the nonparametric Kruskal-Wallis analysis.

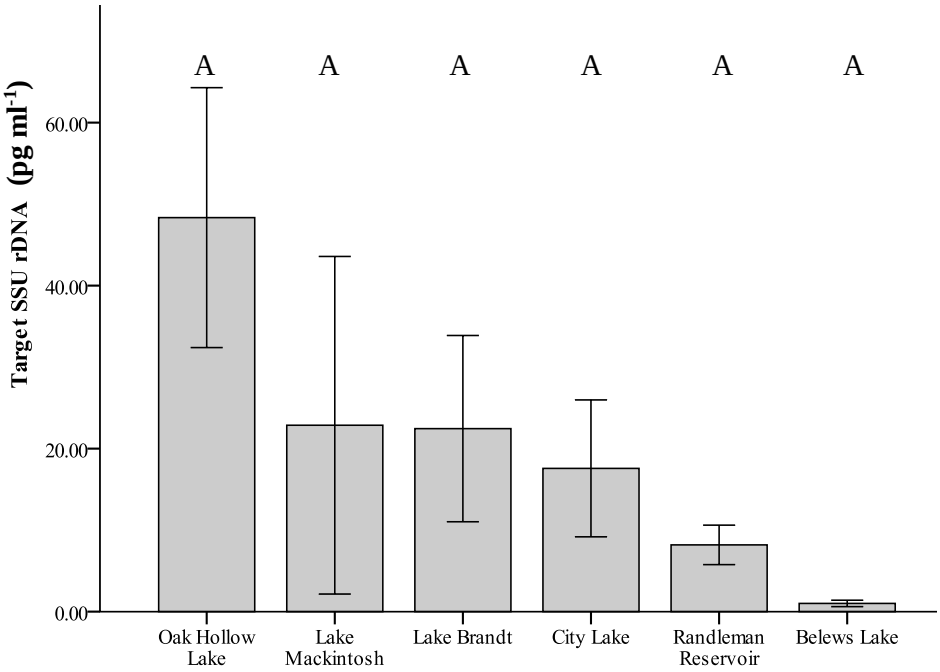


**Figure 15. Mean Total Cyanobacteria Target SSU rDNA ± SE.** Similar letters indicate lakes that show no significant differences in mean total target SSU rDNA based on the nonparametric Kruskal-Wallis analysis.

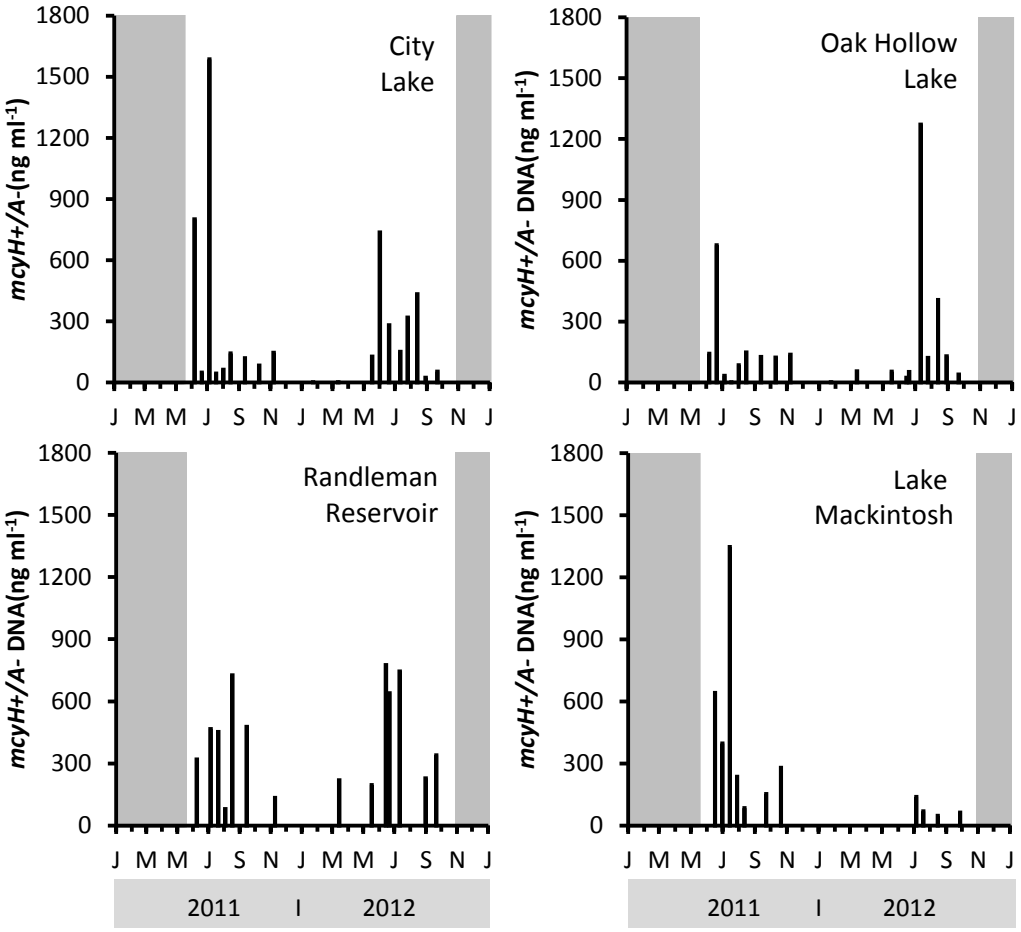


**Figure 16.** ***McyH+/A*- DNA Abundance for Samples Taken between June 2011 and September 2012.**

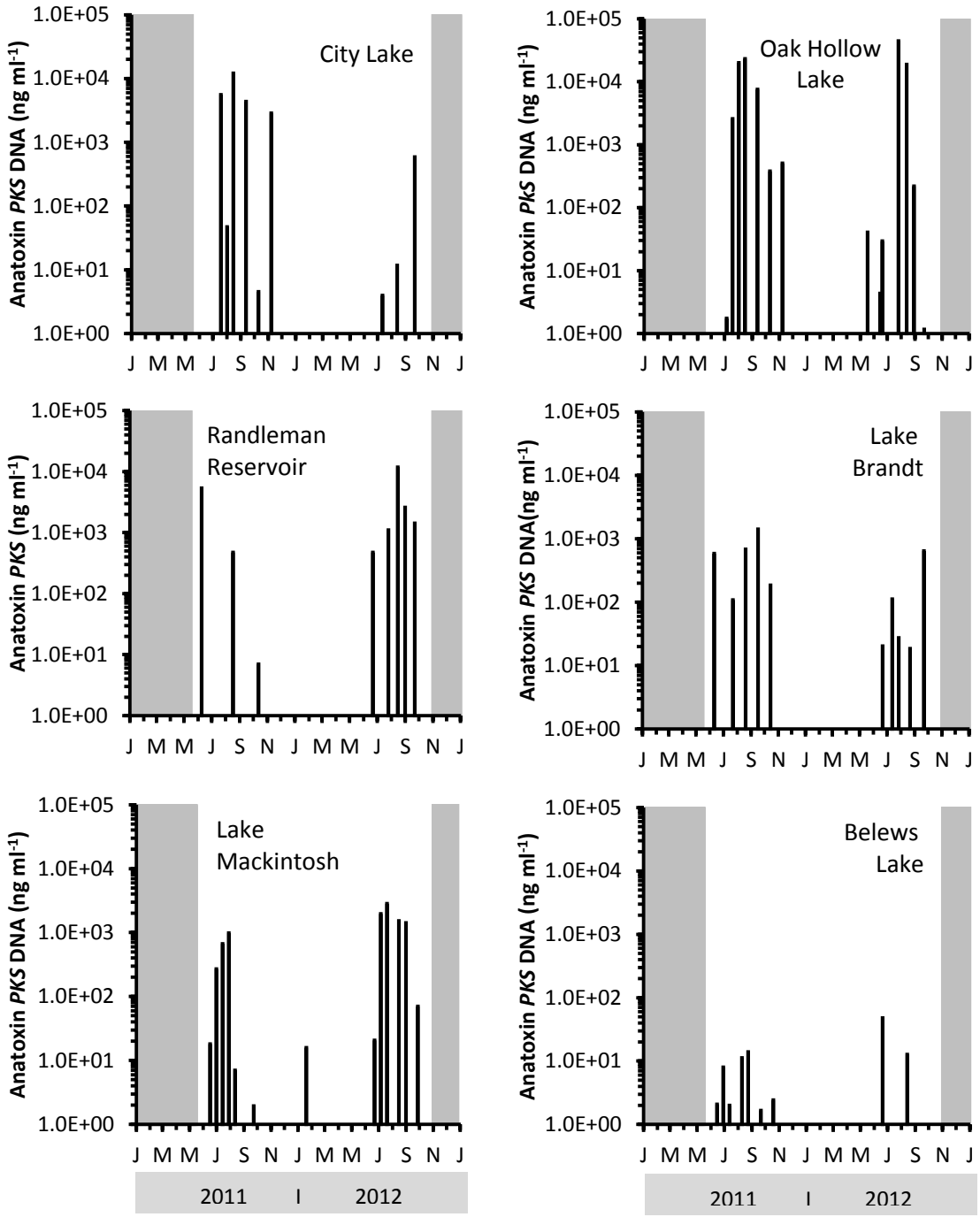


**Figure 17. Anatoxin *PKS* DNA Abundance for Samples Taken between June 2011 and September 2012.**

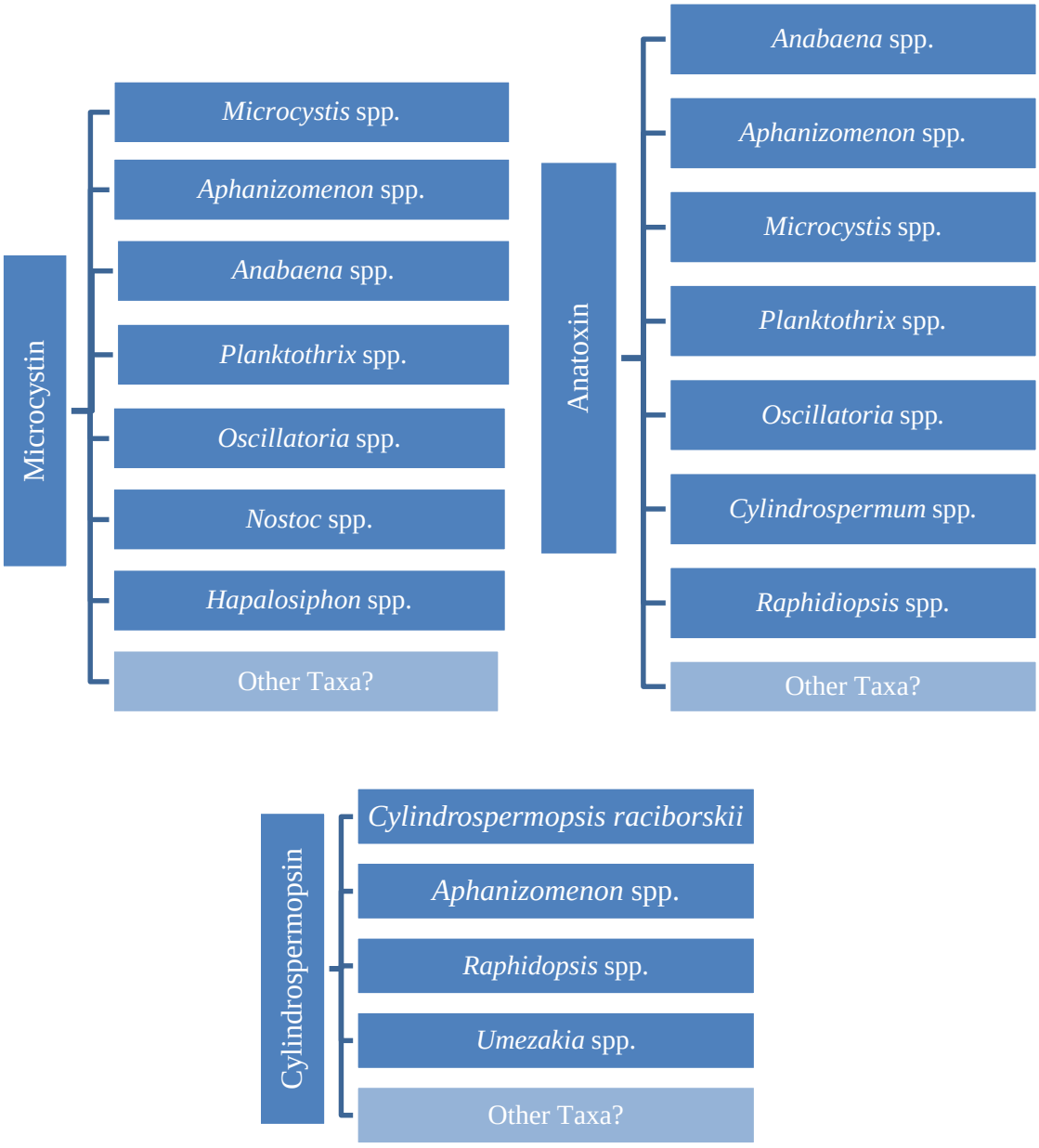


**Figure 18. Relationship Trees Representing Cyanobacteria Genera and Cyanotoxins that May Be Produced.** (Derived from WHO 2003; Fristachi and Sinclair 2008)